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Entomopathogenic nematodes as potential control agents of termites in citrus in Benin

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Contents

1

General introduction

1.1 BACKGROUND

Termites are a large and diverse group of insects classified in an order of their own, the Isoptera. They were first presented at the Royal Society of London in 1781 by Henry Smeathman (Howse, 1970). There are approximately 3,500 species of termites (Engel 2011). Africa is by far the richest continent in termite diversity (Eggleton, 2000). The most economically important termite genera in agricultural and forest areas in Africa are *Macrotermes, Allodontermes, Amitermes, Pseudacanthotermes, Odontotermes, Ancistrotermes, Trinervitermes, Hodotermes* and *Microtermes* (Kumar & Pardeshi, 2011). They dig underground tunnels with a small connection to the aboveground environment (Noirot & Darlington, 2000) through which they reach and attack host plants. Since the major constituent of the diet of termites is cellulose, the insects tunnel through stems and weaken them by consuming the carbohydrates. Eventually the insects cause their collapse or give access to fungi (*Guignardia citricarpa* Kiely) and other disease causing agents (Tristeza virus, the citrus bud mite *Aceria sheldoni*, the citrus nematode *Tylenchulus semipenetrans* Cobb, or the burrowing nematode *Radopholus similis* Cobb*)* (Kumar & Pardeshi, 2011). In Uganda, losses of crops and tree stands ranging from 50 to 100% have been attributed to termite attack (Sekamatte *et al*., 2001). In the semi-arid savannah of Kenya termites were reported to destroy 800-1500 kg/ha of pasture per year (Lepage, 1981). In the countries of Southern Africa, harvester termites are a serious pest of rangeland, removing 60% or more of the standing grass bio-mass during dry years (Coaston, 1958).

In Benin, citrus (*Citrus sinensis* L.) is the second major fruit crop produced and exported after pineapple (FAO, 2001). Regions of high production are located in the southern part of the country, mainly in the departments of Zou, Collines, Oueme, Plateau, Mono, Couffo and Atlantique. Orange trees are the most planted covering an average land of 2.38 ha per citrus grower), followed by mandarin trees (1.73 ha per citrus grower), lemon trees (1.68 ha per citrus grower) and grapefruit trees (0.12 ha per citrus grower). The importance of the production of orange compared to other citrus fruits is due to its importance in the diet of Muslims, especially during Lent of Muslim and it is being exported to Nigeria, a

neighbouring country. The average annual income per producer from citrus is estimated to 1,437,293 CFA francs (2191 euro) for oranges, 143,070 CFA francs (218 euro) for lemon and 57,500 CFA francs (88 euro) for mandarin (ESCiP-Benin, 2012). Citrus harvested areas have increased over the years from 1,470 ha in 1961 to 5,000 ha in 2012. However, in spite of the adoption of new improved varieties, yields were lower than expected, decreasing from 72,624 hg/ha in 1961 to 28,000 hg/ha in 2012 (FAOSTAT, 2014). Citrus growers in different places complain about damages caused to citrus trees by fruit flies, biting butterflies, caterpillars and termites. The latter being the most damaging (ESCiP-Benin, 2012). Since termites make openings to the outside only at an advanced stage of the invasion, citrus growers are frequently unaware of their presence until it is too late (Kumar & Pardeshi, 2011). The losses vary according to harvest times, from 25% (in March-July) to 50% (in November-January) (UEMOA, 2008).

To reduce damage caused by these insects, several control methods, cultural, botanical and chemincal, are used against citrus pests. Among these methods, only the chemical control (use of synthetic insecticides like fipronil, deltametrin, dimethoate and fenitrothion) is mostly used by citrus growers. Although intensive pesticide use has increased crop production, the long-term and highly concentrated application of pesticides may contaminate the yield of crops and pose a serious danger to the agro-ecosystem and human health (Rola & Pingali, 1993). Due to increasing concerns about these side effects, there has been great interest in finding other methods of controlling termites and reducing the use of chemicals (Grace, 1997). Biological control of the pest using entomopathogenic nematodes (EPN) may constitute a promising alternative to chemical controls (Kaya & Koppenhöfer, 2004).

Entomopathogenic nematodes are known since 1923 with the description of *Aplectana kraussei* Steiner, 1923, now *Steinernema kraussei* Travassos, 1927 (Nguyen & Hunt, 2007). Although representatives of nearly 40 nematode families have been isolated from soil inhabiting insects throughout the world, only two families, Steinernematidae Travassos, 1927 and Heterorhabditidae Poinar, 1976, are widely used in biological control for soil-dwelling stages of many insect pests, being safe to most non-target organisms and to the environment

(Kaya & Koppenhöfer, 2004; Ehlers, 2005). Several species of these genera have shown to be an effective pest management tool for both agricultural and urban Integrated Pest Managements (IPM) systems (Shapiro-Ilan *et al.*, 2002). Tamashiro (1968) was the first to propose the use of EPN against termites and many other researchers pursued this approach. Although Epsky and Capinera (1988) and Mauldin and Beal (1989) reported the lack of success of EPN against subterranean termites in laboratory and field studies, the use of these worms against subterranean termites recently regained some interest (Yu *et al.*, 2006, 2008; Ibrahim & Abd El-Latif, 2008; Shahina *et al*., 2011).

Taking into consideration the characteristics of EPN, the VLIR-UOS Own Initiative 2010 project 'Ecologically Sustainable Citrus Production in Benin' has initiated studies on the biodiversity of EPN and their possible use in Benin as control agents for termites. Within the project most of the work is focused on *Macrotermes bellicosus* (Smeathman), the most damaging termite species in citrus in southern Benin; another key termite species, *Trinervitermes occidentalis* (Sjöstedt), was studied in comparison.

1.2 ENTOMOPATHOGENIC NEMATODES

1.2.1 Introduction

Entomopathogenic nematodes are ubiquitous roundworms of the Phylum Nematoda found in nearly all environments throughout the world. Of the nearly 40 nematode families that are associated with insects, only 2, *viz.* Steinernematidae and Heterorhabditidae, are widely used in biological control (Kaya & Koppenhöfer, 2004). They are associated with mostly species-specific symbiotic bacteria: *Steinernema* spp. carry symbiotic bacteria of the genus *Xenorhabdus*, while *Heterorhabditis* spp. carry bacteria of the genus *Photorhabdus* (Boemare, 1996). The bacteria are gram-negative Enterobacteriaceae (Boemare *et al*., 1993), vectored by the nematodes while infecting the host insect. Together, nematodes and their bacterial symbionts are able to kill their insect hosts in a short period of time, usually 24-48 h. The relationship with insects ranges from phoretic or commensal to obligate parasitism and

pathogenesis (Grewal *et al.*, 2005). Out of these *ca.* 40 families, seven receive attention in the field of biological control, *viz.* Mermithidae (Order: Mermithida), Tetradonematidae (Order: Stichosomida); Allantonematidae, Phaenopsitylenchidae and Sphaerulariidae (Order: Tylenchida) along with Heterorhabditidae and Steinernematidae (Order: Rhabditida) (Kaya & Koppenhöfer, 2004). Only the latter two families are getting attention as control agents of insects and are produced commercially by various companies around the world (Stock & Hunt, 2005). Entomopathogenic nematodes offer several advantages over chemical pesticides for termite management including their effective searching abilities, safety to non-target organisms and the environment, good capacity for mass production and compatibility with agricultural chemicals (Kaya & Koppenhöfer, 2004; Ehlers, 2005). In addition to the above-mentioned attributes, they are safe to most non-target organisms and to the environment. No evidence exists for a mammalian pathogenicity (Boemare *et al*., 1996; Ehlers & Hokkanen, 1996). EPN are easily sprayed with standard equipment (Georgis & Kaya, 1998). They can be combined with almost all chemical control compounds and other biological pesticides. They are amenable to genetic selection for desirable traits, and are exempt from registration in many countries (Ehlers, 2005). Soil is the natural reservoir of EPN offering excellent conditions for nematode survival and activity (Gaugler, 1988). The opportunity to use EPN is promising because more than 90% of insect pests spend part of their life cycle in the soil. By the 1980s, fuelled by an enormous infusion of resources from government and industry, research on EPN rapidly expanded. The search for new species of nematodes that could provide effective control of a persistent pest – and be marketed as such – was on (Adams & Nguyen, 2002).

1.2.2 Taxonomic status of entomopathogenic nematodes

Within the Heterorhabditidae and Steinernematidae, species belonging to three genera *viz.* *Heterorhabditis*, *Steinernema* and *Neosteinernema*, have the potential or are being used as biocontrol agents.

The systematic position of EPN species in *Steinernema* and *Heterorhabditis* following the classification of De Ley and Blaxter (2002) is:

CLASS CHROMADOREA Inglis, 1983
Sub class Chromadorea Pearse, 1942
ORDER RHABDITIDA Chitwood, 1933
Suborder Tylenchina Thorne, 1949
Infraorder Panagrolaimomorpha De Ley and Blaxter, 2002
Superfamily Strongyloidoidea Chitwood and McIntosh 1934
Family Steinernematidae Chitwood and Chitwood, 1937
Genus *Steinernema* Travassos, 1927
Genus *Neosteinernema* Nguyen and Smart, 1994

Suborder Rhabditina Chitwood, 1933
Infraorder Rhabditomorpha De Ley and Blaxter, 2002
Superfamily Strongyloidea Baird, 1853
Family Heterorhabditidae Poinar, 1976
Genus *Heterorhabditis* Poinar, 1976

More than 90 species of Steinernematids and Heterorhabditids have been described to date (86 Steinernematids and 18 Heterorhabditids) and these plus 1 species of *Neosteinernema* is now accepted as valid species (Hunt, 2007; Lewis & Clarke, 2012; Shapiro-Ilan *et al*., 2014).

1.2.3 Life cycle

The life cycle of EPN includes eggs, four juvenile stages and an adult stage. The third juvenile stage is a free-living, parasitic stage, known as the infective juvenile (IJ) that searches for susceptible hosts in the soil environment. The IJs of *Steinernema* and *Heterorhabditis* species retain in their gut the symbiotic bacteria of the genus *Xenorhabdus* and *Photorhabdus*, respectively (Campbell & Lewis, 2002). Upon finding a host, the IJ enters through natural openings (mouth, anus or spiracles) (Figure 1.1) and then penetrates into the haemocoel where it releases the mutualistic bacterium from its bacterial vesicle (*Steinernema*) or intestine (*Heterorhabditis*) (Campbell & Lewis, 2002). Some heterorhabditids are able to penetrate inter-segmental membranes of insect cuticle by using an anterior tooth (Bedding & Molyneux, 1982). Whilst multiplying rapidly in nutrient rich insect haemolymph, these bacteria produce toxins that cause septicaemia killing the host within 24-48 hours (Bedding &

Molyneux, 1982). The bacteria digest the cadaver and provide food for nematodes; they also produce antibiotics and other noxious substances that protect the host cadaver from other microbes in the soil (Webster *et al.,* 2002). Eventually the IJ develops into the adult stage (Figure 1.1) and continues its reproduction through one or more generations depending on conditions within the host (Poinar, 1990).

A notable difference between steinernematids and heterorhabditids lies in the reproduction strategy. In steinernematids, reproduction is amphimictic in all generations (Poinar, 1990). The steinernematid IJ develops to adult males or females of the first generation. Most of the eggs from these adult females hatch and the emerging juveniles develop through the different life stages to become adult males or females of the second generation (Dix *et al*., 1994). One *Steinernema* species, i.e. *S. hermaphroditum,* thus far has been found to be an exception; this species may reproduce via hermaphroditism (Stock *et al*., 2004).The life cycle of heterorhabditids is almost similar to the one of steinernematids. The difference between both genera lies in the fact that the first generation of *Heterorhabditis* species is automictic consisting of self-fertile hermaphrodites, while subsequent generations are amphimictic having males and females. Individual hermaphrodites of *Heterorhabditis* lay up to 1000 eggs, which develop into second generation males and females; the first generation hermaphrodites also retains about 500 eggs, which develop into IJ via *endotokia matricida* (Wang & Bedding, 1996). Second generation females also lay 6 to 10 eggs that develop into another generation of adults, but they also retain another 30 eggs within the nematode body which develop into IJ via *Endotokia matricida.* The third generation female does not lay their eggs. The eggs (50 per female) are kept inside their body and develop all via *endotokia matricida* into IJ (Wang & Bedding, 1996).

In both genera, when the nematodes have exhausted the food resources of the host cadaver (usually after two generations), the late second stage juveniles cease feeding. They incorporate a pellet of bacteria in the bacterial chamber / intestine and moult to third stage juveniles (IJ). The IJ leave the cadaver and search for new hosts to start a new life cycle. Infective juveniles do not feed but can survive in soil for several months. The cycle from

entry of IJ into a host until emergence of new IJ is dependent on temperature and varies for different EPN species and strains. It generally takes about 6-18 days at temperatures ranging from 18 to 28°C in *Galleria mellonella* (Poinar, 1990).

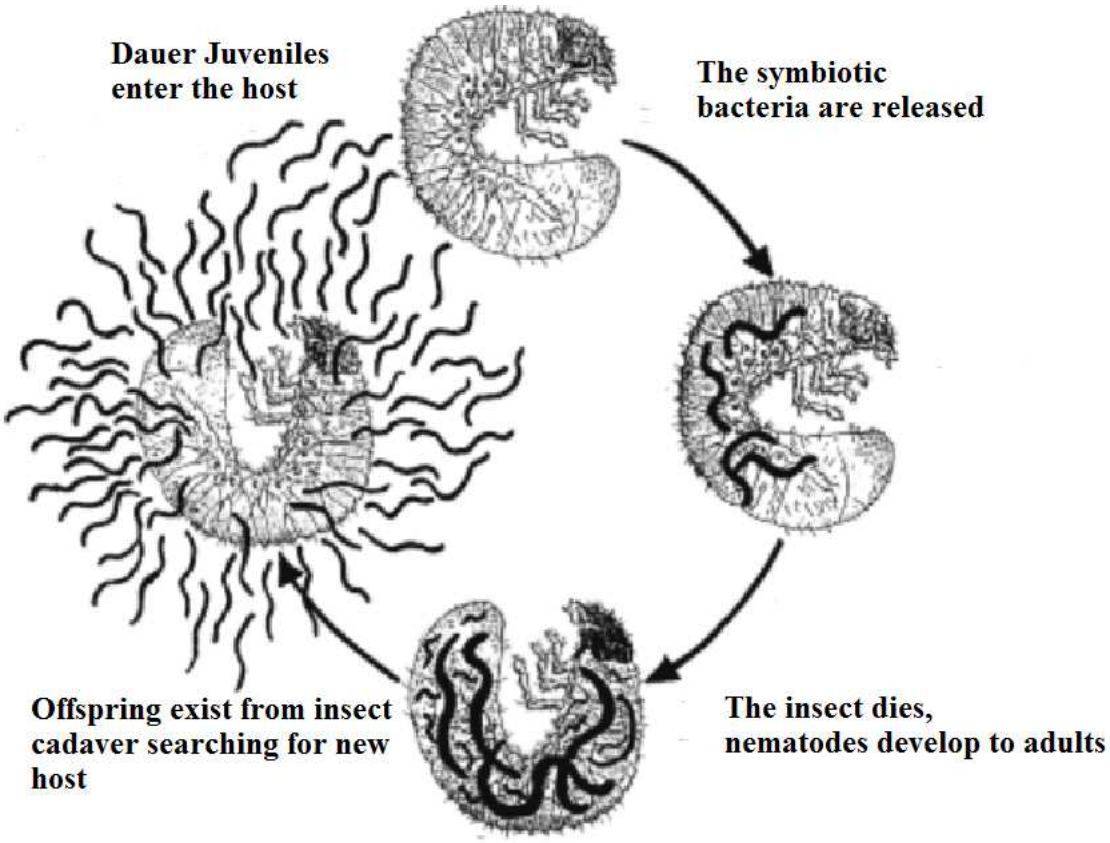

Figure 1.1 Life cycle of entomopathogenic nematodes in a scarabaeid beetle larva (Ehlers, 2001)

1.2.4 Symbiotic bacteria

Xenorhabdus and *Photorhabdus* are two genera of bacteria that symbiotically associate with EPN. *Xenorhabdus* species are found associated with nematodes of the Steinernematidae, while *Photorhabdus* spp. associate only with the genus *Heterorhabditis* (Boemare, 2002). Both genera contain motile gram-negative, non-spore forming facultative anaerobic rods belonging to the Enterobacteriaceae (Boemare, 2002). The bacteria-nematode symbiotic association forms the basis for the nematode's pathogenicity and effectiveness as a biocontrol

agent (Ehlers & Hokkanen, 1996). Both partners benefit from the association. On the one hand the nematodes provide a safe means of transport for the bacteria from one insect host to another into the nutrient rich insect haemolymph and, in some associations, inhibit the insect immune response. On the other hand the bacteria deliver assistance in killing the insect host, supply a suitable nutritive medium for nematode growth and reproduction, and suppress competing organisms by the production of antibiotics (Forst & Clarke, 2002). The life of both symbionts comprises two states. A phoretic state where the bacteria are retained in the intestine of non-feeding IJ without any significant multiplication. A vegetative state, when the bacteria overcome the insect hosts defence system and multiply inside the infected insects (Bedding & Molyneux, 1982).

Although *Xenorhabdus* and *Photorhabdus* spp. are similar in numerous characteristics within the Enterobacteriaceae, they differ in several salient features. A primary property distinguishing *Photorhabdus* spp. from *Xenorhabdus* spp. is that the former are luminescent and catalase positive (i.e. they have ability to emit light under stationary-phase culture conditions and in the infected host insect). On the other hand, *Xenorhabdus* spp. have no luminescence and are catalase negative (Boemare, 2002). Both bacterial genera produce phenotypic variant cell types called primary form (phase I) and secondary form (phase II) (Forst & Clarke, 2002).

In general, for each species of EPN there is a specific association with a species or subspecies of the two genera of the symbiotic bacteria. However, some nematode species share the same bacterial species. For example, *Xenorhabdus bovienii* (Akhurst & Boemare) is associated with four species of *Steinernema* (*S. feltiae, S. kraussei*, *S. affinis* and *S. intermedium*) and *Photorhabdus luminescens* (Thomas & Poinar) is associated with two species of *Heterorhabditis* (*H. bacteriophora* and *H. indica*). More rarely, some bacterial species share the same nematode species: for example, *P. luminescens* and *P. temperata* are both associated with the *H. bacteriophora* group (Nguyen *et al*., 2007).

1.2.5 Nematode movement and host location

Entomopathogenic nematodes have been reported to search for and kill soil-dwelling insect pests (Jackson & Brooks, 1995). These nematodes employ different foraging strategies to locate and infest the host. Based on their host-finding strategies EPN have traditionally been classified as either 'cruisers' or 'ambushers'. Species that do not clearly fall in either category are classified as 'intermediates' (Lewis *et al.*, 2006). Nematodes adopting the cruiser method are thought to move actively through the soil in search of a host. These nematodes are more likely to respond to host volatiles and host cues, since they are capable of moving through the soil towards the host over an extended area (Lewis *et al.*, 1993; Grewal *et al*., 1994a). Juveniles actively cruising through the soil are more likely to encounter non-moving subterranean hosts than IJ of the immobile ambusher type. Nematodes adopting the ambush strategy remain in the same area and adopt a "sit and wait" approach to encountering a new host. Juveniles using this approach to host finding are most likely to encounter an actively moving host near or on the surface of the soil. One of the most common behaviours ambushers display is known as nictation. This involves the juvenile staying on the surface of the substrate and standing on its tail. The nematode then waves its body back and forth in order to attach to a passing host (Campbell & Gaugler, 1993). Ambushers are less likely to respond to distant host cues such as carbon dioxide (Lewis *et al.*, 1993). *Steinernema glaseri, H. downesi, H. megidis* and *H. bacteriophora* are classed as cruiser foragers (Grewal *at al*., 1994a). *Steinernema carpocapsae* is classed as an ambusher forager whereas *S. feltiae* is classed as an intermediate (Lewis, 2002).

1.2.6 Environmental factors affecting nematode survival and infectivity

The success of an insect control program using EPN largely depends on the environmental conditions under which the nematodes are applied (Grewal *et al.*, 1994b, 2006). Abiotic factors in the soil environment, such as soil texture, moisture, temperature, aeration, pH and UV radiation can affect EPN survival (Kaya, 1990); biotic factors such as competition between different nematode species, competition with other insect pathogens, nematophagous

fungi, and natural enemies such as collembolans, mites, tardigrades and predatory nematodes (Koppenhöfer, 2000) could prevent the EPN from realizing their full potential as bio-insecticides.

 Influence of abiotic factors

Temperature is one of the key factors affecting the infectivity of nematodes (Finnegan *et al*., 1999; Hazir *et al*., 2001); however, optimum temperatures for infection and reproduction vary among nematode species and strains (Grewal *et al*., 1994b). Some species such as *H. indica*, *S. glaseri* and *S. riobrave* are relatively heat tolerant and can maintain efficacy at temperatures of 29°C and above, whereas others, such as *S. feltiae*, *H. megidis* and *H. marelatus*, are more cold tolerant maintaining efficacy at 15°C and below (Grewal *et al.*, 1994b; Kung *et al.*, 1991; Shapiro & McCoy, 2000). When studying the effect of temperature on eight species of EPN, Grewal *et al*. (1994b) noted that *S. feltiae* infected and established in *G. mellonella* larvae between 8 and 30°C and reproduced between 10 and 25°C, while *S. riobrave* infected and established between 10 and 39°C and reproduced between 20 and 35°C. *Steinernema carpocapsae* possesses an intermediate and narrow thermal reproductive niche breadth of 20-30ºC and has a reproduction optimum at 25ºC. Earlier research by Khlibsuwan *et al*. (1992) reported that *S. carpocapsae* was able to locate a host insect and was unaffected by temperatures below 33°C; however, locomotion and infectivity were impaired at 35 and 37°C. Recent studies carried out by Ma *et al*. (2013) showed that following exposure at -5° C for 8 h, the isolates HQA87 of *S. ceratophorum*, HML39 and All of *S. carpocapsae* and ZZ68 of *H. indica* showed good cold tolerance with survival of IJ of 89, 87, 82, 83%, respectively, whereas the isolates JJJ163 of *S. ceratophorum* and HDT75 of *S. longicaudum* showed poor tolerance with survival of IJ of 6.3 and 5.5%, respectively. The authors showed that all these isolates survived heat at 40° C for 2 h with 100% survival of the IJ.

Moisture is another important factor affecting EPN activity in soil (Kaya, 1990). Georgis and Gaugler (1991) found that soil moisture was positively related to EPN efficacy in the control of white grubs in turfgrass. Studies such as those of Lacey and Unruh (1998) and Grant and Villani (2003) showed that soil moisture strongly influenced the survival, movement and

pathogenicity of EPN. However, too much moisture may cause oxygen deprivation and restrict movement (Kaya, 1990). Entomopathogenic nematodes do not survive rapid desiccation in laboratory experiments (Womersley, 1990; Kung *et al*., 1991) but can persist for considerable lengths of time in dry soil if allowed to dry out gradually (Kung *et al*., 1991). Desiccation significantly reduces the survival of nematodes and is one important factor affecting commercial use of the nematodes at every stage, from their mass production to application in the field (Strauch *et al*., 2004).

Soil texture plays an important role in the movement and dispersal of EPN. Movement is impaired in heavy clay soils and movement improves as the percentage of silt and clay in the soil decreases (Kaya, 1990). Likewise, the thickness of the organic matter on the surface of the soil can also negatively affect EPN movement into the soil after application (Georgis & Gaugler, 1991). Since nematodes are aerobic organisms, low oxygen availability can reduce their survival (Wharton, 1986). Soil pH can also have an effect on nematode survival and infectivity; pH of 10 or higher is likely to be detrimental (Kung *et al.*, 1990).

UV radiation is detrimental to EPN (Gaugler & Boush, 1978). Infective juveniles of EPN are very susceptible to UV radiation and die quickly when exposed to light at a frequency of around 300 nm for more than a few minutes. Direct sunlight is tolerable for up to half an hour before adverse effects on infectivity and survival of nematodes are observed. *Steinernema carpocapsae* is susceptible to short UV (254 nm), but is more resistant to longer UV (366 nm); the species may therefore be more effective in biocontrol applications involving exposed surfaces (Gaugler & Boush 1978). Thus, if EPN are being applied to the soil surface, it is best to apply nematodes to soil in the evening or early morning hours. Alternatively, efficacy can be improved, and exposure to UV radiation avoided, through sub-surface application (Cabanillas & Raulston, 1995).

- *Influence of biotic factors*

Many natural enemies of nematodes have been identified as parasites/pathogens, predators, competitors or antagonists. Their impact is largely unknown under field conditions (Kaya, 2002) but laboratory results suggest that antagonists such as nematophagous fungi may

influence EPN dynamics (Koppenhöffer *et al.*, 1996). Nematophagous fungi are found in a wide range of soil habitats throughout the world (Gray, 1988). The two basic types are predatory or trapping fungi and endoparasitic fungi. Other organisms in soil can have a positive or negative influence on the movement, dispersal and survival of EPN. Vertical dispersal of *Steinernema* spp. can be increased by earthworms (Shapiro *et al.*, 1993). In some cases insect hosts can be responsible for phoresis of EPN for up to two days post-infection (Downes & Griffin, 1996). This sort of phoresis by infected flying adult hosts could cause widespread dispersal further than the EPN would be capable of on their own (Kaya, 1990). Unintentional transport by humans is also thought to be responsible for long range dispersal of EPN in cargos and ships' ballast (Downes & Griffin, 1996).

1.2.7 Distribution and occurrence of entomopathogenic nematodes in Africa

Entomopathogenic nematodes have a global distribution and have been isolated from every continent and are found in virtually every terrestrial ecosystem (Hominick, 2002). Antarctica is the only continent from which they have not been recorded (Griffin *et al.*, 1991), whereas the African continent remains largely a fertile field for EPN exploration. At the end of the 20th century, only *S. feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1992 (Egypt) and *S. karii* Waturu, Hunt & Reid, 1997 (Kenya), along with *H. bacteriophora* Poinar, 1976 (Kenya and South Africa), *H. indica* Poinar, Karunakar & David, 1992 (Egypt and Kenya) and *H. taysearae* Shamseldean, El-Sooud, Abd-Elgawad & Saleh, 1996 (Egypt) had been recorded for this continent (Peters, 1996; Shamseldean *et al.*, 1996; Waturu, 1998). More recent surveys in Africa revealed several new species: *S. yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005, *S. ethiopiense* Tamiru, Waeyenberge, Hailu, Ehlers, Půža & Mráček, 2012 (both from Ethiopia), *S. khoisanae* Nguyen, Malan & Gozel, 2006, *H. safricana* Malan, Nguyen, De Waal & Tiedt, 2008, *S. citrae* Malan, Knoetze & Moore, 2011, *Steinernema tophus* n. sp. Cimen, Lee, Hatting, Hazir & Stock, 2014 and *H. noenieputensis* n. sp. Malan, Knoetze & Tiedt, 2014 (from South Africa), and *S. cameroonense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 and *S. nyetense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 (both from Cameroon) (Nguyen *et al.*,

2006; Nguyen & Hunt, 2007; Malan *et al.*, 2008; Malan *et al*., 2011; Kanga *et al*., 2012; Tamiru *et al.*, 2012, Cimen *et al*., 2014; Malan *et al*., 2014). New strains of *H. indica* and *H. bacteriophora* were reported from Kenya and Egypt (Stack *et al*., 2000; Hominick, 2002), new strains of *S. yirgalemense, S. karii* and *S. weiseri* Mráček, Sturhan & Reid, 2003 were found in the Central Rift Valley Region of Kenya (Nyasani *et al*., 2008) and a population of *S. yirgalemense* was detected in South Africa (Malan *et al*., 2011). In Ethiopia, the dominant species detected was *S. yirgalemense* next to two isolates of *H. bacteriophora* (Mekete *et al*., 2005). Kanga *et al*. (2012) reported from Cameroon new strains of *H. baujardi* Phan, Subbotin, Nguyen & Moens, 2003, a species originally described from Vietnam and later also recorded from Brazil. New strains of *H. indica* and *H. sonorensis* were reported from Benin. The latter was the most dominant and recorded for the second time since its description from the Sonora desert in Mexico (Chapter 2).

1.2.8 Formulation and application

Entomopathogenic nematodes can be reared and mass-produced on artificial chemically known solid/liquid monoxenic media or by *in vivo* cultivation using suitable host insect. The IJ need to be formulated in materials that guarantee survival for a period necessary to market the nematode product. In order to increase their shelf life and efficacy, EPN have been formulated commercially in various carriers such as sponges, vermiculite, liquid concentrates, alginate gels and water-dispersible granules (Grewal, 2000; Koppenhöfer, 2000). They are applied as IJ in aqueous suspensions by use of various irrigation systems, sprayers, or injection techniques. These nematodes have also been shown to be effective when applied in their infected-host cadavers (Jansson *et al*., 1993). In comparison with application in aqueous suspension, laboratory studies have indicated that application of infected cadavers may result in superior nematode dispersal (Shapiro & Glazer, 1996), infectivity (Shapiro & Lewis, 1999) and survival (Perez *et al*., 2003).

1.3 TERMITES

1.3.1 Introduction

Termites (Isoptera: Termitidae) are designated as soil ecological engineers due to their diversified feeding, foraging and nesting activities and subsequent impact on the bioavailability of soil resources to other trophic levels in soil profiles (Lavelle *et al*., 1997; Romero-Lopez *et al*., 2010). However, a number of species can be important pests in man-made ecosystems, such as agricultural and forest plantations (Cowie *et al*., 1989). Their vast biomass alone makes them an important consideration in tropical and sub-tropical ecology (Josens, 1983). Termites are a diverse and varied group comprising several feeding groups, including wood feeders, grass harvesters and soil feeders, which exert different ecological effects on ecosystems (Josens, 1983; Donovan *et al*., 2002). There are approximately 3,500 species of termites (Engel, 2011), which are now classified into ten families (Engel et *al.*, 2009). These families are the Mastotermitidae, Termopsidae, Hodotermitidae, Archotermopsidae, Stolotermitidae, Kalotermitidae, Stylotermitidae, Rhinotermitidae, Serritermitidae, and Termitidae. Most of the known genera and species that damage crops, trees, and rangeland belong to the Termitidae (Pomeroy *et al.*, 1991; Mitchell, 2002) that consists of four subfamilies: Nasutitermitinae, Termitinae, Apictotermitinae and Macrotermitinae. Over 90% of the termite damage in agriculture, forestry, and urban settings is attributed to members of the Macrotermitinae (Pomeroy *et al*., 1991; Mitchell, 2002).

Surveys conducted in citrus in southern Benin revealed the occurrence of two families of termites, the Rhinotermitidae and the Termitidae. The latter being the most abundant with 8 species all belonging to the subfamily Macrotermitinae included *Amitermes guineensis* (Sands), *Ancistrotermes crucifer* (Sjöstedt), *Anguilitermes truncatus* (Sjöstedt), *Cubitermes* sp., *Macrotermes bellicosus* (Smeathman), *Microcerotermes progrediens* (Silvestri), *Pericaptritermes* sp., *Trinervitermes occidentalis* (Sjöstedt), *Trinervitermes* sp., *Trinervitermes trinervius* (Rambur). Only one species, *Coptotermes intermedius* (Silvestri) was found for Rhinotermitidae (ESCiP-Benin, 2011). Citrus growers distinguished mainly two species: *Macrotermes bellicosus* causing damage to citrus trees and another species

Trinervitermes occidentalis causing damage to crops (ESCiP-Benin, 2011). *Macrotermes bellicosus*, generally referred to as 'war-like termite'. Besides the negative effects on citrus, *M. bellicosu*s nest-mounds get in the way of agricultural machinery and sometimes have to be levelled with bulldozers, thus increasing the cost of mechanized farming.

1.3.2 General termite biology

Termites are truly social insects. They possess the three main characteristics to being eusocial: i) there is cooperative brood care in their colonies, ii) they have overlapping generations, iii) and they are divided into different castes which perform different tasks within the colony (Wilson, 1971). A termite colony is a family of individuals all living together. It generally has an inanimate and an animate part. The inanimate part is the structures built by individuals within which they live; the animate part is the individuals living within the colony. Sometimes the inanimate part of the colony is just a few tunnels, but often it is a very extensive and sophisticated structure. Some very specialized termite species do not build their own nests but live exclusively inside other termites' buildings; well-known examples being *Serritermes serrifer* (Serritermitidae) and *Inquilinitermes* spp. These are called 'inquilines', a term that also applies to those termite species that are able to build their own nests but are facultative termitaria invaders. Termites nests may also house microbes, plants, invertebrates and vertebrates, which are called 'termitophiles' or 'termitariophiles', depending on whether they are associated to the host termites or to the termitaria itself.

The animate part of a colony can be grouped into morphological 'castes', which can be reproductive (king, queen, and their reproductive offspring) or sterile (workers and soldiers). The queen is generally the only egg-laying individual in the colony. Once there are many workers to help the queen, her only task is to produce a tremendous number of offspring. The queen has the longest life, spanning on average of 25 years. The other types of termite's life span vary from 12 to 24 months. The king is her consort and his only duty appears to mate with her regularly (Korb, 2008). The alates are winged reproductives preparing to leave the nest in order to swarm, to pair and to start new colonies. Workers, on the other hand, never

leave the nest except to forage for food. They are the mainstay of the colony, and their roles are numerous. They forage for food and water, build and repair colony structures, and tend the immatures, alates, the king and the queen. Soldiers have only one job that consists of defending the colony, and particularly the queen and the king. As with other biological systems, this simple description of caste structure is complicated by some species that have no soldiers and others that have no workers. Neotropical Apicotermitinae (Termitidae) termites do not possess soldiers, and Kalotermitidae and Termopsidae (traditional sense or Archotermopsidae in the new classification) do not possess true workers; whereas *Macrotermes bellicosus* and *Trinervitermes occidentalis* (Termitidae) possess both soldiers and workers (Boomsma, 2009; Bignell *et al*., 2011).

The life cycle is similar in all termites and scientifically known as a hemimetabolous (incomplete metamorphosis) life cycle. Colonies produce winged reproductive 'alates', often at the start of the rainy season in drier or seasonal habitats (Martius *et al*., 1996). From time to time, mainly after a rain, the alates emerge from their nest. They land on the ground or on a piece of dead wood and pair up: one male with one female. They shed their wings and mate. The pair then found a colony; the female lays her eggs either in the soil or in dead wood. The eggs hatch to nymphs which are tended by the two parents (Figure 1.2). The nymphs grow into workers that begin to tend young, to build colony structures and to forage for food. Slightly later in the colony's development, soldiers are produced. When the colony has reached maturity, alates are produced again and the cycle continues.

1.3.3 *Macrotermes bellicosus* and *Trinervitermes occidentalis*

Macrotermes bellicosus (Figure 1.3) is a fungus-growing termite that builds large epigeous nests (cathedral form) from fine particles (clay) cemented with saliva (Boutton *et al*., 1983). It has a wide geographical distribution occurring in savannahs ranging from West- to East-Africa, but is absent from rain forests (Ruelle, 1970; Pomeroy, 1978). It is the largest species known, with queens measuring about 110 mm long, workers about 36 mm and soldiers being slightly larger than the latter. They supplement their diet by growing fungi (Ruelle 1970).

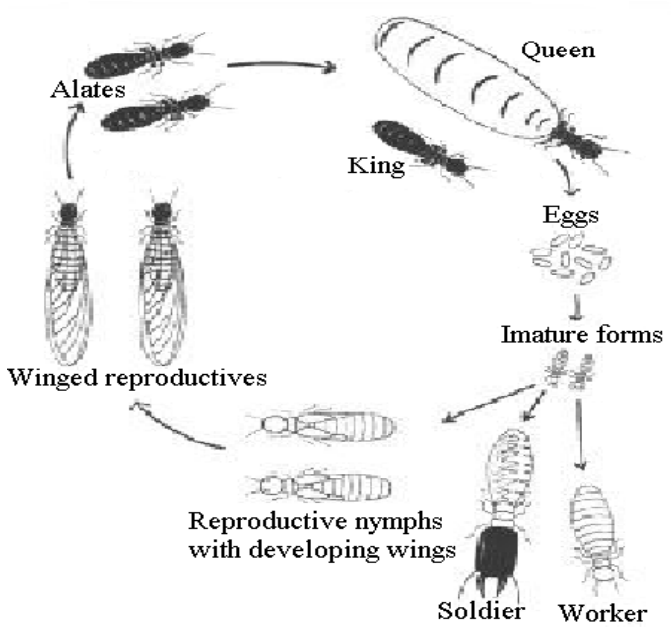

Figure 1.2 Typical life cycle of the subterranean termite. The queen lays eggs year-round whereas the release of winged reproductives is an annual event. (Source: Horwood & Eldridge, 2005).

They chew up wood and digest the nutrients that can be taken in to grow fungi. The rest is passed and used for gardening. Species of the genus *Macrotermes* (Macrotermitinae) cultivate symbiotic basidiomycete fungi of the genus *Termitomyces* that digest plant-derived persistent ingredients such as lignin and tannin (Collins, 1981). *Macrotermes* species construct nests with epigeal mounds and extensive underground gallery systems (Collins, 1979) by soil composition modification due to non-random selection of soil particles (Abe *et al.*, 2009). Growing rooms are built for the purpose of raising this food source for the colony. For an optimal growth *Termitomyces* fungi cultivated by *M. bellicosus* require constant temperatures of 30◦C (Lüscher 1961).

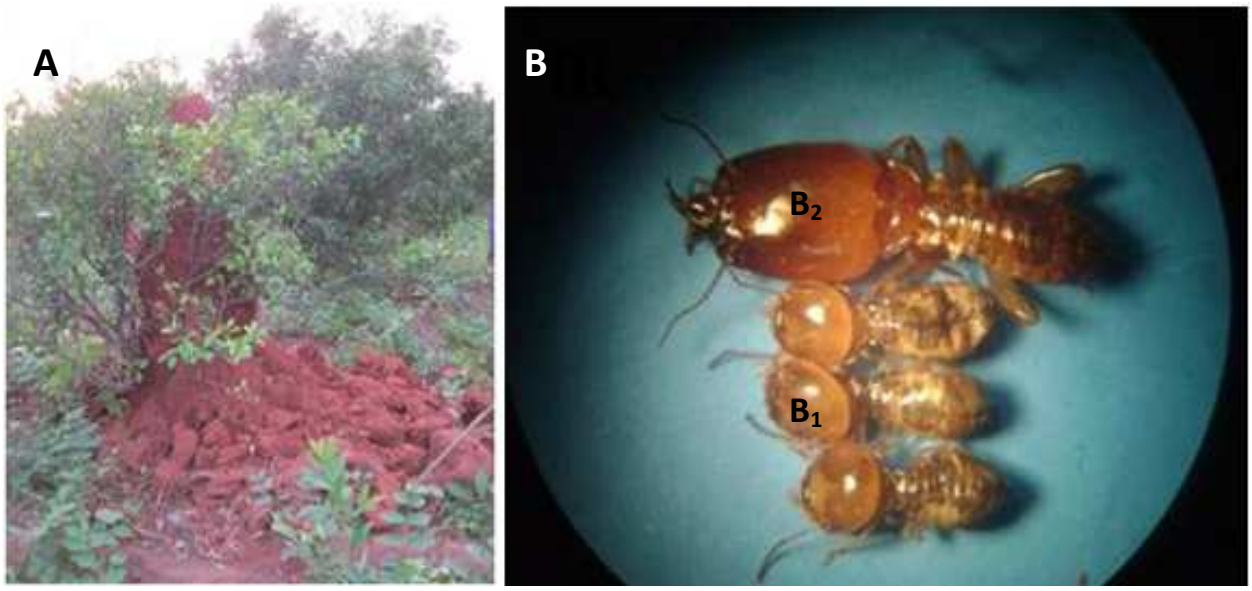

Figure 1.3 *Macrotermes bellicosus* mound squeezing a citrus tree (A), worker (B_1) and soldier (B_2)

Trinervitermes occidentalis (Figure 1.4) is a grass-feeder termite that is common in African Savannah. It forages for dead dry standing grass, incorporates also faeces in its mound structure, which exhibits higher carbon content and very high cation exchange capacity (CEC) as compared to the adjacent top-soil (Ndiaye *et al.*, 2003). Mounds are constructed with soil cemented with salivary material, but galleries and chambers are lined with faecal material. Centre mound temperatures may reach a peak of 40 °C (Adam, 1993).

In Benin, until citrus trees are large, crops (maize, cassava, groundnut or bean) are grown for food within the plantation during the first 3-6 years. The crops are subject to attacks by *T. occidentalis* that feed on their stems, leaves and straws. Therefore, the co-occurrence of both *M. bellicosus* and *T. occidentalis* in citrus orchards affected seriously farmers' incomes. This latter termite species was therefore included in our study.

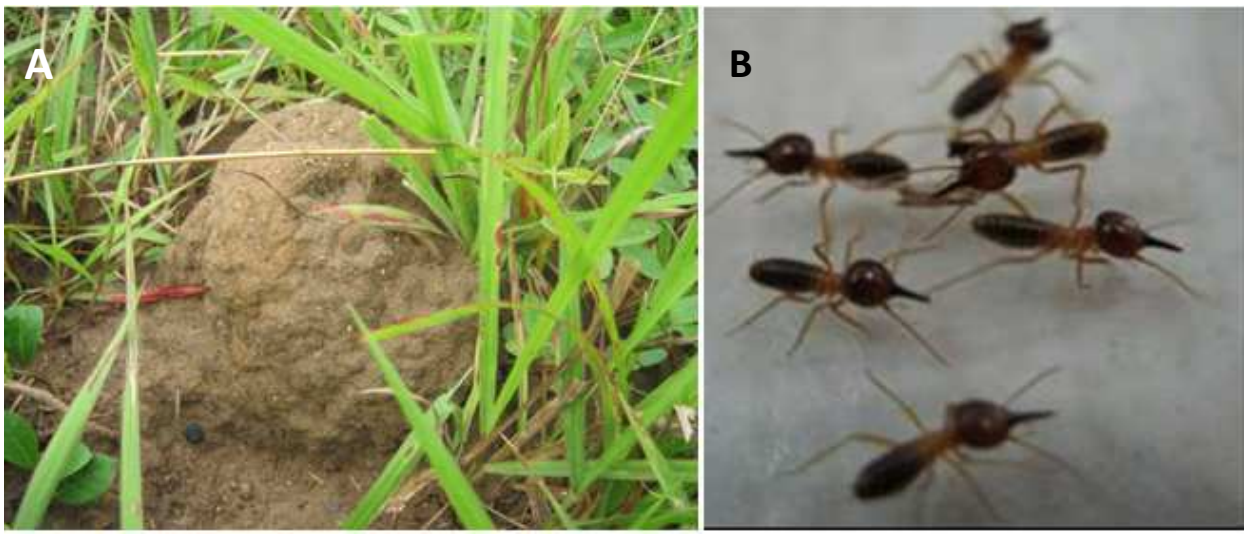

Figure 1.4 *Trinervitermes occidentalis* mound (A) and soldiers (B)

1.3.4 Overview on biological control of subterranean termites using entomopathogenic nematodes

Subterranean termites occupy the same soil habitat as EPN, but they appear not particularly susceptible to infection by EPN (Fuiji, 1975). Few studies have addressed the potential of nematodes to control termites. In an early report, Bedding and Stanfield (1981) reported that large colonies of the Australian genus *Mastotermes* could be killed using *Heterorhabditis* spp. by injecting the nematodes directly into the infested eucalyptus trunks. Danthanarayana and Vitharana (1987) demonstrated in Sri-Lanka that live-wood termites could be killed by injecting large quantities of EPN directly inside the branches of tea plants; unfortunately, no follow up study was done and this approach was never implemented. Mortality exceeding 95% was recorded by Georgis *et al.* (1982) for both *Zootermopsis* sp. and *Reticulitermes* sp. within 3 days after laboratory exposure to *S. carpocapsae*; termites were also found to carry infection back to their colonies. Epsky and Capinera (1988) concluded that *S. carpocapsae* showed potential for control of *Reticulitermes tibialis* (Banks) in laboratory and field trials. In spite of these findings, Epsky and Capinera (1988) and Mauldin and Beal (1989) reported the

lack of success using nematodes against subterranean termites in laboratory and field studies. Both Reese (1971) and Fuiji (1975) failed to suppress *Coptotermes formosanus* (Shiraki) due to the fact that infected termites were successfully walled off by nest mates to protect the colony.

More recently, Wang *et al.* (2002), studied the virulence of *S. carpocapsae* (Breton), *S. riobrave* (TX), *H. bacteriophora* (HP88), and *H. indica* (Coimbatore) to *Reticulitermes flavipes* (Kollar) and *C. formosanus*. In Petri dish tests, the EPN species were all effective against *C. formosanus* at a dose of 400 nematodes termite^{-1}. Later, Yu *et al.* (2008) showed that *S. carpocapsae* (Mexican 33 strain), *S. feltiae* (UK76 strain) and *H. bacteriophora* (HP88 strain) can infect and kill the desert subterranean termite *Heterotermes aureus* (Snyder) in laboratory conditions. These species can also develop and reproduce in termite cadavers and emerge as IJ. Furthermore, *S. riobrave* was found to cause over 75% mortality of workers of three termite species, *viz. Gnathamitermes perplexus* (Banks), *H. aureus* and *R. flavipes* (Yu *et al.*, 2006; 2010). Recently, Manzoor (2012) reported *H. bacteriophora* and *S. carpocapsae* to be effective against workers of *R. flavipes.*

Since the potential of EPN to control termites has been demonstrated, further investigation is justified. This research should focus on the biology, ecology and physiology of the EPN-termite interactions in laboratory as well as on field. It should provide more accurate guidelines to the biological control of termites with EPN.

1.4 AIMS

The overall aim of this thesis is to develop a sustainable alternative to termite control by synthetic chemicals. Because EPN are not yet commercially available in Benin, additional attention should be given to the EPN production at cottage industry level; the potential of formulated insect cadavers infected with EPN against termite pests should be examined.

The main objectives of this study are to gain more understanding on the ecological and biological control characteristics of indigenous EPN in Benin. These objectives translate into the following specific objectives:

(i) To acquire fundamental knowledge on EPN distribution and diversity in southern Benin based on molecular, morphological/morphometric approaches and multivariate of ecological parameter analyses. (Chapter 2).

(ii) To explore the biological characteristics of indigenous EPN (Chapter 3, 6).

(iii) To obtain deeper understanding of nematode-termite interactions (Chapter 4, 5).

(iv) To develop an integrated termite control involving traditional practice and EPN (Chapter 6).

1.5 THESIS OUTLINE

This thesis contains seven chapters.

Chapter 1 gives a general introduction. It illustrates the context of this study, presenting an overview of EPN and termites and their interaction, and explaining the main aims of this work and their logical flow based on literature and with focus on Africa, Benin.

Chapter 2-5 presents the results. These chapters have been drafted according to requirements for manuscripts in SCI-indexed journals.

Chapter 2. **First record on the distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in Southern Benin**. It presents the occurrence of two *Heterorhabditis* species and describes their distribution in agricultural and natural habitats.

Chapter 3. **Characterization of biocontrol traits of heterorhabditid isolates from South Benin targeting the termite pest *Macrotermes bellicosus***. It shows the results of the screening of these 32 Beninese isolates for their tolerance to heat, desiccation and hypoxia, and the effect of these environmental extremes on their virulence in laboratory

tests. The screening was completed with observations on the virulence against *M. bellicosus* in semi-field conditions.

Chapter 4. **Effectiveness of *Heterorhabditis* isolates from Southern Benin for the biocontrol of the subterranean termite, *Macrotermes bellicosus* (Isoptera: Macrotermitinae), in laboratory trials**. It reports on laboratory (sand column) tests for comparative host finding ability of heterorhabditid isolates. The virulence of EPN against the citrus termite pests *M. bellicosus* was also investigated.

Chapter 5. **Comparative susceptibility of *Macrotermes bellicosus* and *Trinervitermes occidentalis* (Isoptera: Termitidae) to EPN from Benin.** It demonstrates the difference in virulence between three indigenous EPN species (*Heterorhabditis sonorensis, H. indica* and an undescribed *Steinernema* sp.) towards two castes (worker and soldier) of *M. bellicosus* and *T. occidentalis* in relation to penetration efficiency of IJ and production rates of IJ in both termite species/castes. The abilities of the nematodes to parasitize termites at different concentrations of IJ and to recycle were also investigated, as well as the repellent effect of nematodes on termites.

Chapter 6. **Influence of pesticides, soil temperature and moisture on entomopathogenic nematodes from southern Benin and their potential to control underground termite nest population** shows the impact of three pesticides (fipronil, sulfur and glyphophate), soil temperature and soil moisture on the virulence of three indigenous EPN species (*Heterorhabditis sonorensis, H. indica* and an undescribed *Steinernema* sp.). An integrated termite control involving EPN and traditional practice is also presented.

Chapter 7 contains the **General discussion**, which integrates the principal findings of Chapters 2-6, and future prospects.

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2

First record on the distribution of entomopathogenic nematodes (Rhabditida: Steinernematidae and Heterorhabditidae) in southern Benin

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ABSTRACT

For the first time, surveys of entomopathogenic nematodes (EPN) were conducted in five departments in the Guinean zone of Southern Benin. Out of 84 prospected sites and 280 collected soil samples from agricultural and natural vegetation, 26 (30.95%) and 32 (11.43%) were positive for EPN, respectively. Identification of the EPN was based on analyses of sequences of the ITS rDNA region and morphological/morphometric investigations. Two species were found, *viz. Heterorhabditis sonorensis* and *H. indica.* This is the first record of *H. sonorensis* since its description from the Sonora dessert in Mexico. *Heterorhabditis sonorensis* was the most common species, showing a preference for semi-closed habitats such as citrus orchards, other fruit production fields and woodland with soils with sand and organic matter content ranging between 53.6-89.5% and 0.1-4.7%, respectively, and a pH from acidic (4) to neutral (7.1). Entomopathogenic nematodes were not recovered from crop fields (maize, cassava, groundnut, and bean) and soil samples with less than 50% sand content. *Heterorhabditis indica* was associated with citrus orchards and fruit fields on sand to sandy clay soils, with pH slightly acidic (pH = 5.4-6.4), but not with woodland. Discriminant analysis identified five major environmental variables, *viz.* longitude, organic matter content and texture (silt, sand and clay content) to be the most important abiotic factors determining the occurrence of EPN in soil from Southern Benin. Using these parameters, redundancy analysis revealed *H. sonorensis* and *H. indica* to prefer soils with high sand or organic matter content located in the more eastern longitude. No significant difference was observed in EPN species preferences taken individually, in terms of studied ecological parameters.

2.1 INTRODUCTION

Entomopathogenic nematodes (EPN) are known since 1923 with the description of *Aplectana kraussei* Steiner, 1923, now *Steinernema kraussei* (Steiner, 1923) Travassos, 1927 (Nguyen & Hunt, 2007). Although nearly 40 nematode families have been isolated from soil inhabiting insects throughout the world, only two families, *viz*. Steinernematidae Travassos, 1927 and Heterorhabditidae Poinar, 1976, are of major interest in agriculture because of their potential in regulating insect populations, particularly insect pests with a soil-dwelling phase (Kaya & Gaugler, 1993). They are associated with symbiotic bacteria belonging to the genus *Xenorhabdus* (*Steinernema* spp.) and *Photorhabdus* (*Heterorhabditis* spp.), respectively (Boemare *et al.*, 1993). Entomopathogenic nematodes along with their bacterial symbionts are able to kill their insect hosts in a short period of time, usually within 24 to 48 h.

Numerous surveys have provided evidence of the omnipresence of these nematodes. Entomopathogenic nematodes have been isolated from many types of natural and managed habitats in a wide variety of soils throughout the world (Hominick, 2002); Antarctica is the only continent from which they have not been recorded (Griffin *et al*., 1991). The African continent remains largely a fertile field for EPN exploration. At the end of the 20th century, only two *Steinernema* species, *viz*. *S. feltiae* (Filipjev, 1934) Wouts, Mráček, Gerdin & Bedding, 1992 (Egypt) and *S. karii* Waturu, Hunt & Reid, 1997 (Kenya), and three *Heterorhabditis* species, *viz. H. bacteriophora* Poinar, 1976 (Kenya and South Africa), *H. indica* Poinar, Karunakar & David, 1992 (Egypt and Kenya) and *H. taysearae* Shamseldean, El-Sooud, Abd-Elgawad & Saleh, 1996 (Egypt) had been recorded for this continent (Peters, 1996; Shamseldean *et al*., 1996; Waturu, 1998). More recent surveys in Africa revealed several new species: *S. yirgalemense* Nguyen, Tesfamariam, Gozel, Gaugler & Adams, 2005, *S. ethiopiense* Tamiru, Waeyenberge, Hailu, Ehlers, Půža & Mráček, 2012 (both from Ethiopia), *S. khoisanae* Nguyen, Malan & Gozel, 2006, *H. safricana* Malan, Nguyen, De Waal & Tiedt, 2008, *S. citrae* Malan, Knoetze & Moore, 2011, *Steinernema tophus* n. sp. Cimen, Lee, Hatting, Hazir & Stock, 2014 and *H. noenieputensis* n. sp. Malan, Knoetze & Tiedt, 2014 (from South Africa), and *S. cameroonense* Kanga, Trinh, Waeyenberge, Spiridonov, Hauser & Moens, 2012 and *S. nyetense* Kanga, Trinh, Waeyenberge, Spiridonov,

Hauser & Moens, 2012 (both from Cameroon) (Nguyen *et al.,* 2006; Nguyen & Hunt, 2007; Malan *et al.,* 2008; Malan *et al*., 2011; Kanga *et al.,* 2012; Tamiru *et al.*, 2012; Cimen *et al*., 2014; Malan *et al.,* 2014). New strains of *H. indica* and *H. bacteriophora* were reported from Kenya and Egypt (Stack *et al*., 2000; Hominick, 2002), new isolates of *S. yirgalemense*, *S. karii* and *S. weiseri* Mráček, Sturhan & Reid, 2003 were found in the Central Rift Valley Region of Kenya (Nyasani *et al*., 2008) and a population of *S. yirgalemense* was detected in South Africa (Malan *et al*., 2011). In Ethiopia, the dominant species detected was *S. yirgalemense* (6.3%) next to two isolates of *H. bacteriophora* (0.7%) (Mekete *et al*., 2005). Kanga *et al*. (2012) reported from Cameroon new strains of *H. baujardi* Phan, Subbotin, Nguyen & Moens, 2003, a species originally described from Vietnam and later also recorded from Brazil.

Thus far, no study has been published on the EPN distribution in Benin. However, within the framework of the VLIR-UOS Own Initiative 2010 project on *Ecologically Sustainable Citrus Production in Benin*, the potential of indigenous EPN as an environmental friendly alternative control agent to the commonly used synthetic chemicals is being investigated. In this respect, surveys for EPN were conducted in 2010 and 2011 throughout the southern part of Benin in woodlands, crop fields and fruit fields.

The current contribution presents the first records of EPN from Benin. It reports the occurrence of two *Heterorhabditis* species in southern Benin and their distribution in agricultural and natural habitats.

2.2 MATERIALS AND METHODS

2.2.1 Site description and sampling characterization

The Republic of Benin is situated in West Africa between the latitudes 6º10’N and 12º 25’N and longitudes 0º45’E and 3º55’E. Three climate zones can broadly be distinguished (Akoègninou, 2004): (i) the Northern zone located between 9°45’ – 12°25’N, (ii) the transition zone located between 7°30’ – 9°45’N, and (iii) the Southern zone located between

6°25' – 7°30'N. Soil samples for our study were collected from the Southern zone where the climate is Guinean with two rainy seasons alternating with a long dry season (December-February). Two surveys of EPN were carried out during the rainy season in September 2010 and August 2011. In the prospected area (Figure 2.1), the mean annual rainfall varies from 1048.3 to 1604 mm; the mean annual temperature ranges from 26 to 28°C and the annual relative humidity from 75.5 to 81.7% (Table 2.1).

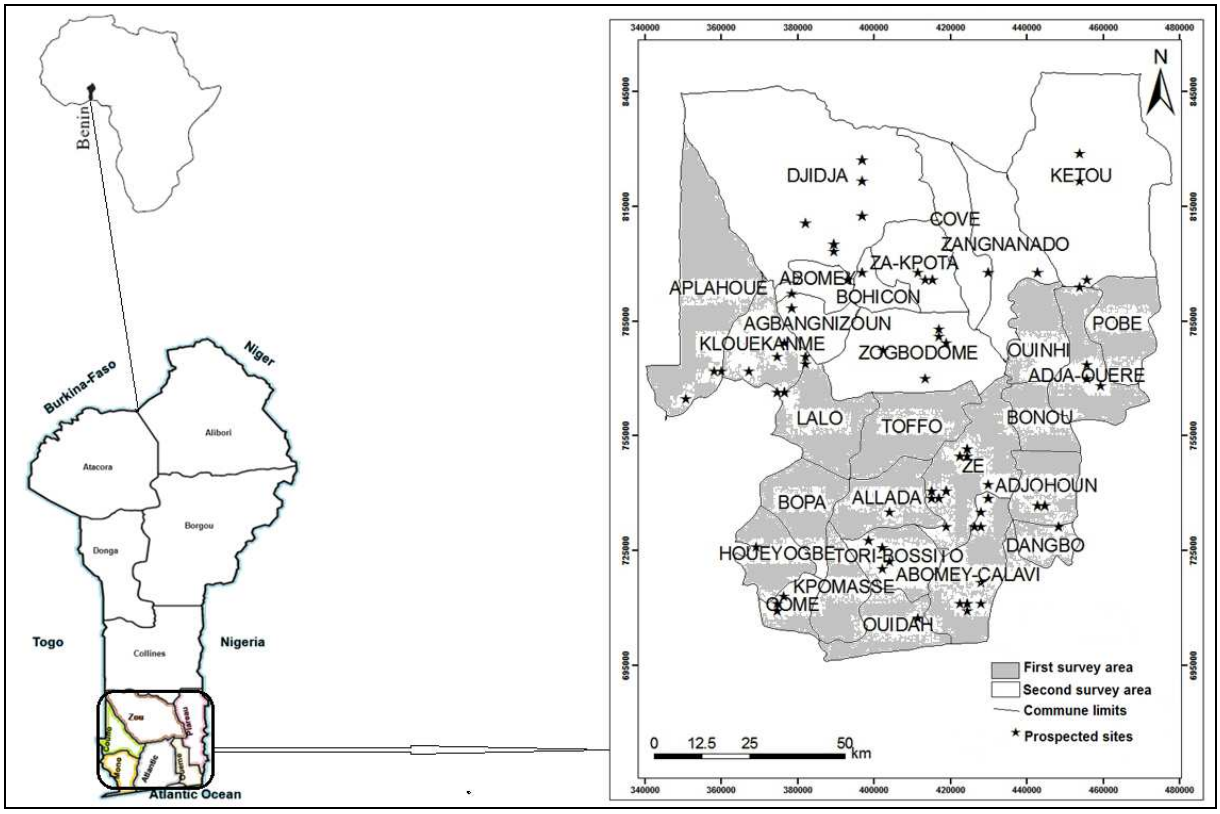

Figure 2.1 Map showing the sampling sites in Southern Benin

Regarding the context of this study, the first survey was mainly orientated towards citrus orchards (orange, lemon and grapefruit) with a high incidence of insect populations. During the second survey, samples were also taken in fields next to citrus orchards and considered other fruit fields (mango, cashew, avocado, guava, banana, oil palm), annual and perennial crop fields (maize, cassava, groundnut, bean), and woodland (teak, acacia, eucalyptus). A total of 280 soil samples were collected from 84 sites distributed over 28 communities. At

each site, a total of 3 – 4 samples, at least 10 m apart, were taken. Each soil sample (approximately 1.5 kg) consisted of a composite of 3 – 5 cores randomly taken in a 9-m^2 area, always close to a tree or plant, at a depth of 0-15 cm and using a hand trowel. Samples were placed in polyethylene bags to prevent water loss and kept in coolers (*ca* 15°C) during transit to the laboratory.

At each site, data on sampling location, habitat (vegetation), longitude, latitude and altitude were recorded. For each sampling site, a subsample (*ca* 300 g) was analysed for the following physical and chemical characteristics: pH, organic matter, sand, silt, and clay content. Soil samples were processed at the Soil Analysis Laboratory of the Faculty of Agronomy Sciences, University of Abomey Calavi, Benin.

2.2.2 Nematode isolation

Entomopathogenic nematodes were recovered from soil samples using the insect baiting method (Bedding & Akhurst, 1975). Within a week after the sampling, a subsample of *ca* 350 cm^3 was transferred to a 360-cm^3 plastic container to which five larvae of *Galleria mellonella* were added as bait. The containers were inverted and kept in the dark at ambient temperature of 27 ± 2°C. Five days after incubation, the dead larvae were removed, rinsed successively with alcohol (70%) and water. Dead larvae that exhibited signs of infection with EPN, i.e. placid soft odourless larvae with either light to dark brown or reddish colour, were washed and placed in modified White traps (White, 1927). The larvae on the White trap were checked for emergence of nematodes after one week and thereafter daily. All nematodes emerging from dead larvae of the same sample were bulked and considered as one isolate. In the case of negative results, the isolation was repeated once to confirm results of the first observation.

To verify the pathogenicity of the collected nematodes and to establish new cultures, emerging nematodes were used to infect fresh *G. mellonella* larvae (Kaya & Stock, 1997). Then, dead larvae were collected and separately placed on a White trap. The emerged nematodes were collected alive in distilled water over a 2-week period and stored at 13°C.

Isolate stocks were maintained on *G. mellonella* larvae being re-inoculated on a 2- month basis.

Table 2.1 Sampled departments in Benin: geographic location and climate characteristics.

Department	Latitude	Longitude	Altitude* (m)	AP	MAT	RH
Atlantique	02° 15′ 00″	06° 40′ 00″	27	1604	28.4	81
Couffo	01° 48′ 06″	06° 57′ 43″	121	1326.5	28.2	75.8
Mono	01° 43′ 00″	06° 38′ 00″	12	1048.3	27.8	81.5
Oueme	02° 36′ 00″	06° 30′ 00″	31	1553.4	28.4	80.8
Plateau	02° 41′ 00″	06° 58′ 00″	75	1305.2	27.6	81.7
Zou	01° 59′ 00″	07° 11′ 00″	121	1114.07	28.2	75.5

*Source: Agence pour la Sécurité de la Navigation Aérienne en Afrique et à Madagascar (ASECNA).

AP = Annual precipitation (mm) ; MAT = Mean annual temperature* (°C) ; RH = Relative humidity* (%)*

2.2.3 Nematode identification

Both molecular and morphological/morphometric approaches were used for the nematode identification, starting with the molecular analysis (Joyce *et al*., 1994; Stock, 2009).

2.2.3.1 Molecular characterization and phylogenetic analysis

Molecular characterization of the isolates was performed by analysis of the ITS rDNA sequences. Genomic DNA was extracted from a single nematode and the ITS regions and the 5.8S rRNA gene of the ribosomal DNA (rDNA) was amplified by PCR according to procedures described by Joyce *et al*. (1994). The obtained 707-bp sequences were compared with all DNA-sequences of *Heterorhabditis* species available in Genbank using the Basic

Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (www.ncbi.com).

An alignment of the ITS1-5.8S-ITS2 sequences was generated using Clustal W (Thompson et *al.*, 1997) and used for phylogenetic analysis. A phylogenetic tree was produced using the Neighbour-Joining method (Saitou & Nei, 1987). The analysis involved 48 nucleotide sequences and was conducted in MEGA5 (Tamura *et al*., 2011). In agreement with Nguyen *et al.* (2010) *Caenorhabditis elegans* (EU131007) was applied as outgroup.

2.2.3.2 Morphological characterization

Nematodes were examined live or heat-killed in 60°C Ringer's solution. The heat-killed nematodes were placed in triethanolamine–formalin (TAF) fixative (Kaya & Stock, 1997) and processed to anhydrous glycerine for mounting (Seinhorst, 1959). Observations were made from live and mounted specimens using an Olympus BX51 microscope equipped with differential interference contrast optics and digital image software (Cell^D Soft Imaging System, Olympus Company, Japan). For morphological characterization of the isolates, 20 second-generation males and 30 IJs were randomly selected from different *G. mellonella* cadavers (Nguyen & Smart Jr, 1995). According to their morphological traits, isolates were placed into similar species-groups using taxonomic criteria suggested by Stock and Kaya (1996) and Hominick *et al*. (1997).

2.2.3.3 Cross-hybridization tests

Cross hybridization tests were carried out on 3.5cm lipid agar plates (Wouts, 1981). These plates were pre-inoculated with 1-2 drops of primary phase of the bacteria (previously extracted from the haemolymph of infested *Galleria* larvae) and incubated for 48h in the dark at 30°C (Phan *et al.*, 2003). The following crossings were performed: *H. indica* Ayogbe1 × *H. indica* LN2, *H. sonorensis* Kpedekpo × *H. taysearae*, *H. sonorensis* Akohoun × *H. taysearae*, *H. sonorensis* Setto1 × *H. taysearae*, *H. sonorensis* Ze1 × *H. taysearae* and *H. sonorensis* Ze3 × *H. taysearae*. For each crossing, 20 males and 20 females of the appropriate isolate were added to the plate and

incubated at 25°C. Two other tests served as controls: a) a virginity/self fertility test in which 20 virgin females were incubated without males on pre-inoculated lipid agar plates (Stack *et al.*, 2000) and b) a self-cross where 20 males and 20 females of the same isolate were incubated together (Phan *et al.*, 2003). The results of crossing between different isolates were considered as valid only if there was no progeny in both the virginity test and the self-cross (Stack *et al.*, 2000). Mating between male and female of the same species should produce fertile offspring (Nguyen, 2007).

Unfortunately, no isolates of *H. sonorensis* were available for crossbreeding tests. Also the Beninese specimens could not be morphologically compared with type material of *H. sonorensis* since type specimens were not available in the nematode collections indicated in the original description.

2.2.4 Data analysis

Diversity patterns among sampling sites were assessed by means of Principal Component Analysis (PCA) performed with environmental variables (latitude, longitude, altitude, habitat, pH, soil texture, silt, sand, clay, and organic matter content of the soil). To scrutinize the ecological trends of EPN isolates, the occurrence of EPN related to the environmental variables was first assessed with their recovery frequency (number positive samples/total number samples) (Liu & Berry 1995), expressed as percentage. Afterwards, Discriminant Analysis (DA) was used to extract which of the environmental variables were the best predictors of presence of EPN in soil samples. These latter variables were used in Redundancy Analysis (RDA) to investigate differences in ecological preferences of the *Heterorhabditis* species found (Van den Wollenberg, 1977). The species matrix data was characterized by EPN species presence/absence and the analysis was conducted on centred response variables because species frequencies do not require standardization. The selected environmental variables were used as explanatory variables. Statistical analyses were performed using STATISTCA 7 for PCA and DA, and XLSTAT 2012 for RDA.

2.3 RESULTS

2.3.1 Identification of isolates

Out of 280 collected soil samples over 84 prospected sites, 32 (11.43%) and 26 (30.95%) were EPN-positive, respectively. Molecular examination revealed that they all belonged to the genus *Heterorhabditis.* Twenty-nine isolates showed 100% sequence similarity with *H. sonorensis* Stock, Rivera-Orduño & Flores-Lara, 2009; 99% with *H. taysearae*; 98% with *H. mexicana* Nguyen, Shapiro-Ilan, Stuart, McCoy, James & Adams, 2004 and *H. floridensis* Nguyen, Gozel, Koppenhöfer & Adams, 2006; and 97% with *H. amazonensis* Andaló, Nguyen & Moino, 2006 and *H. baujardi*. The remainder of the selected isolates shared sequence similarity of 99% with *H. indica.*

In the phylogenetic tree inferred from the ITS sequences (sum of branch length = 660.72; Figure 2.2), the 29 aforementioned isolates clustered with *H. sonoren*sis (bootstrap value = 63%). The pairwise distance comparison (showed below branches) revealed the 29 isolates to differ from *H. taysearae* and *H. mexicana* by one and four bases, respectively. No sequence difference was found between these isolates and *H. sonorensis*. Hence, we concluded that these 29 isolates belonged to *H. sonoren*sis. Likewise, three other isolates (Ayogbe1, Ayogbe2 and Dodji) clustered in a separate group (bootstrap value = 96%). They formed a monophyletic clade with *H. indica* supported by a high bootstrap value of 99% (Figure 2.2). They therefore were considered conspecific with *H. indica*.

The morphology and morphometrics of the isolates were conform to the original description of the species to which they were assigned. Both species are easily separated from each other by the position of the excretory/secretory pore of the male, which in *H. indica*, is located posterior to the basal bulb, whereas in *H. sonorensis* it is usually posterior to the nerve ring at the level of the basal bulb (data not shown). Further, the gubernaculum of *H. indica* is flat, about half the spicule length in size, whereas the gubernaculum of *H. sonorensis* is slightly curved ventrally and about 60% of spicule length (data not shown).

Crossbreeding of *H. indica* Ayogbe1 and *H. indica* LN2 yielded fertile progeny. No progeny was obtained from the crossing between *H. sonorensis* Kpedekpo, *H. sonorensis* Akohoun, *H. sonorensis* Setto1, *H. sonorensis* Ze1, *H. sonorensis* Ze3 and *H. taysearae.*

2.3.2 Spatial distribution of EPN and interrelated variables

2.3.2.1 Diversity patterns of sampled sites

The correlation between features characterizing the environment and major components 1 and 2 (Table 2.2) revealed that the first three components expressed 73.15% of data variability and are sufficient to describe reliably diversity patterns among sampled sites. The first component represented a gradient of decreasing organic matter content (−0.61), pH (−0.64), longitude (−0.89) and latitude (−0.89) with habitat (−0.60) changing progressively from semi-closed habitats (woodland, fruit fields and citrus orchards) to open habitats (crop fields) from left to the right. The second component represented increasing contents of sand (0.94) and decreasing contents of silt (−0.67) and clay (−0.83) from the bottom to the top. The third component represented soil texture (0.89) changing from sand, sandy loam, sandy clay loam and sandy clay from the bottom to the top.

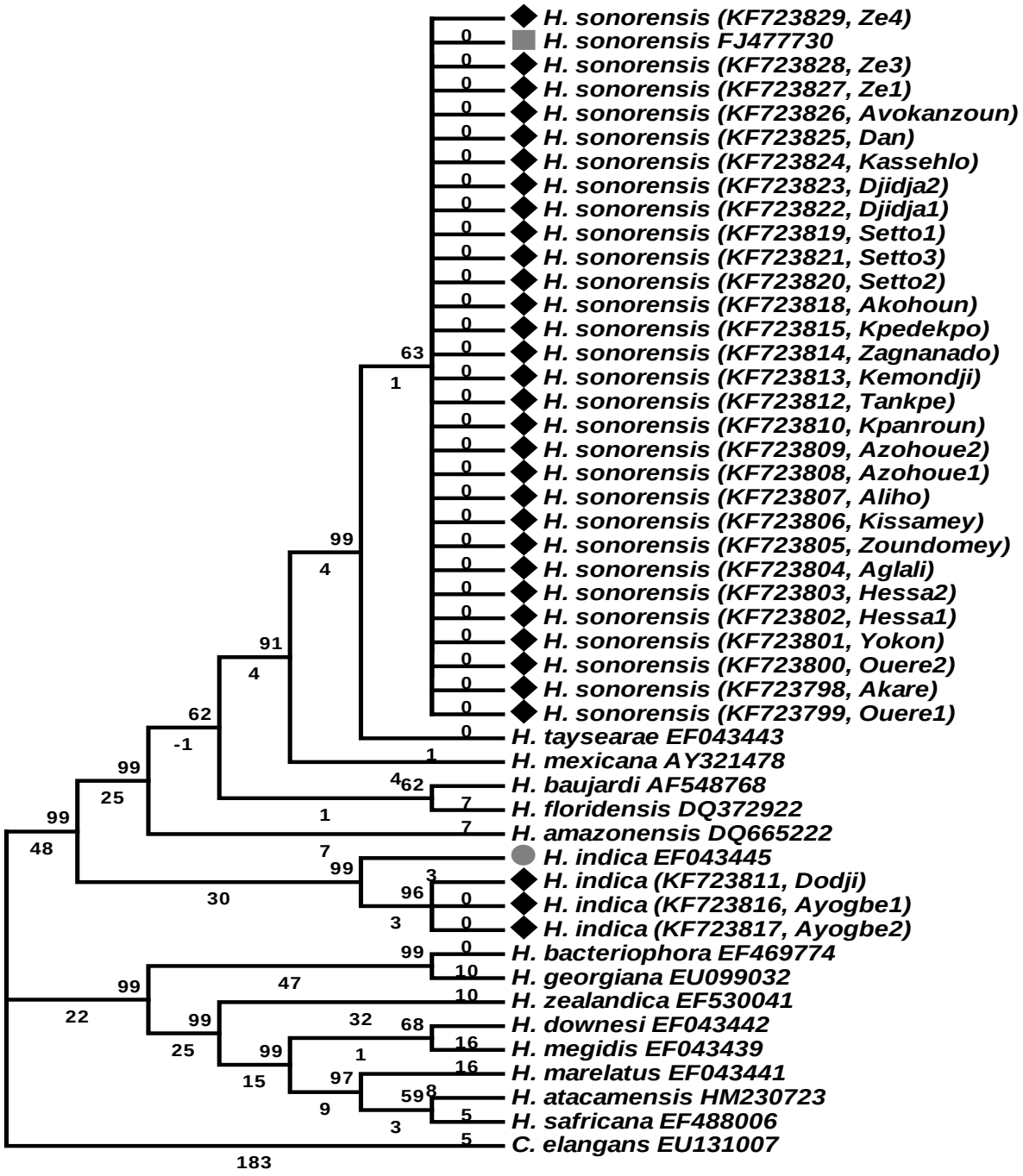

Figure 2.2 Phylogenetic relationships of 15 *Heterorhabditis* species for which ITS sequences are available in Genbank, 32 *Heterorhabditis* strains from Benin, and one outgroup species (*Caenorhabditis elegans*) based on analysis of ITS regions by Neighbour-Joining method. Tree length = 660.72. Numbers above the nodes indicate bootstrap value and numbers below branches represent base differences per sequence. Numbers after species name correspond to (GenBank accession numbers, sample code). ◆ =. *Heterorhabditis* isolates from Benin. ■ = Type strain of *H. sonorensis*. ● = Type strain of *H. indica*

Table 2.2 Correlation between variables characterizing the environment of sampled sites in south Benin and the first three components of the principal component analysis (Bold values indicate parameters dominating principal components 1, 2 or 3).

Variables	Components		
	1	**2**	**3**
Silt	-0.46	**-0.67**	0.51
Sand	0.29	**0.94**	-0.06
Clay	0.01	**-0.83**	-0.45
Soil texture	-0.24	-0.06	**0.89**
Organic matter	**-0.61**	-0.45	-0.32
pH	**-0.64**	0.24	0.14
Altitude	0.45	0.04	0.10
Habitat	**-0.60**	0.18	-0.28
Longitude	**-0.89**	0.35	-0.08
Latitude	**-0.89**	0.35	-0.08
Eigen values variance	3.27	2.56	1.48
Percentage of variance	32.73	25.57	14.85
Cumulative percentage of variance (%)	32.73	58.30	73.15

When soil samples were projected on the plane of components 1 and 2 (Figure 2.3A), samples from the more western longitude and the more southern latitude, with the highest pH and the lowest organic matter, silt, sand and clay content collected in open habitat (crop field) were located at the top left of the quadrant I. Samples from the more eastern longitude and the more north latitude, with the lowest pH, organic matter, silt and clay content, and the highest sand content collected in semi-closed habitat (citrus orchards, fruit fields and woodland) were located at the top right of the quadrant II. Quadrant III contained samples from the more western longitude and western latitude, with the highest pH, organic matter, silt, and clay content and the lowest sand content collected in opened habitat (crop field). When soil samples were projected on the plane of components 1 and 3 (Figure 2.3B), samples from lighter-textured soils were located in quadrants I and II.

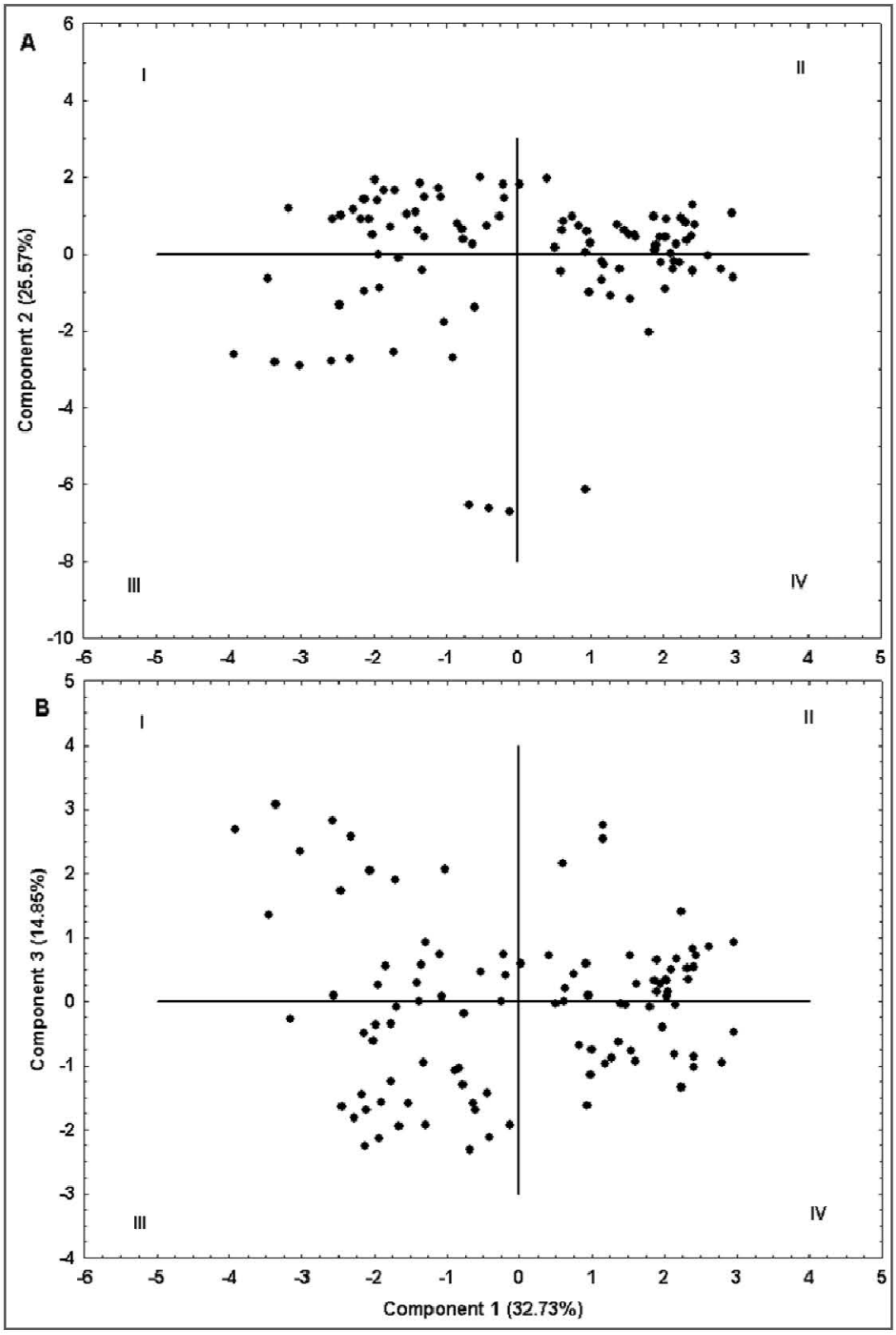

Figure 2.3 Projection of soil samples on the planes defined by the component 1 and 2 (A) and the components 1 and 3 (B) of the principal component analysis of soil samples involving environmental variables (latitude, longitude, altitude, habitat, pH, silt, sand, clay and organic matter content and texture). Dots represent often more than one soil sample.

2.3.2.2 Occurrence of isolates with associated ecological parameters

In terms of recovery frequency, an unequal repartition of EPN isolates was observed (Table 2.3 and Figure 2.4). Entomopathogenic nematodes occurred in all the studied ecosystems, with the exception of crop fields and soils with more than 40% silt or clay content, or less than 50% sand content.

In terms of species diversity, *H. sonorensis* was the most prevalent species found (Figure 2.4) in 90.6% of the positive samples located between latitudes 1º48’ and 2°37’, longitudes 6°25’ and 7°30’ and altitudes between 8 and 252 m. The soil samples positive for *H. sonorensis* had a pH ranging from 4 to 7, a silt content from 1.3 to 30.5%, a sand content from 53.6 to 89.5%, a clay content from 4.5 to 36.4%, and an organic matter content between 0.1 and 4.7%. This species was found at sites with a sandy, sandy clay, sandy loam, or sandy clay loam soil texture and occurred in semi-closed habitats, such as citrus orchards, fruit fields and woodland.

Heterorhabditis indica was found in 9.4% of the positive samples. They were located between latitude 2°15’ and 2°00’ and longitude 6°41’ and 7°17’. It was found associated with citrus orchards and fruit field habitats, at altitudes between 12 − 102 m, in soils with pH ranging from 5.4 to 6.4, silt content from 2.3 to 2.7%, sand content from 77.1 to 83.4%, clay content from 20.6 – 13.9% and organic matter content from 2.4 to 2.9%. The soil textures were sand and sandy clay.

Heterorhabditis sonorensis was found in all sub-ecosystems where *H. indica* was found. However, *H. indica* was not present in sub-ecosystems with sandy loam and sandy clay loam soil types, with pH < 5 located, and altitude > 150 m and latitude below 2°00’, where *H. sonorensis* was present (Table 2.3).

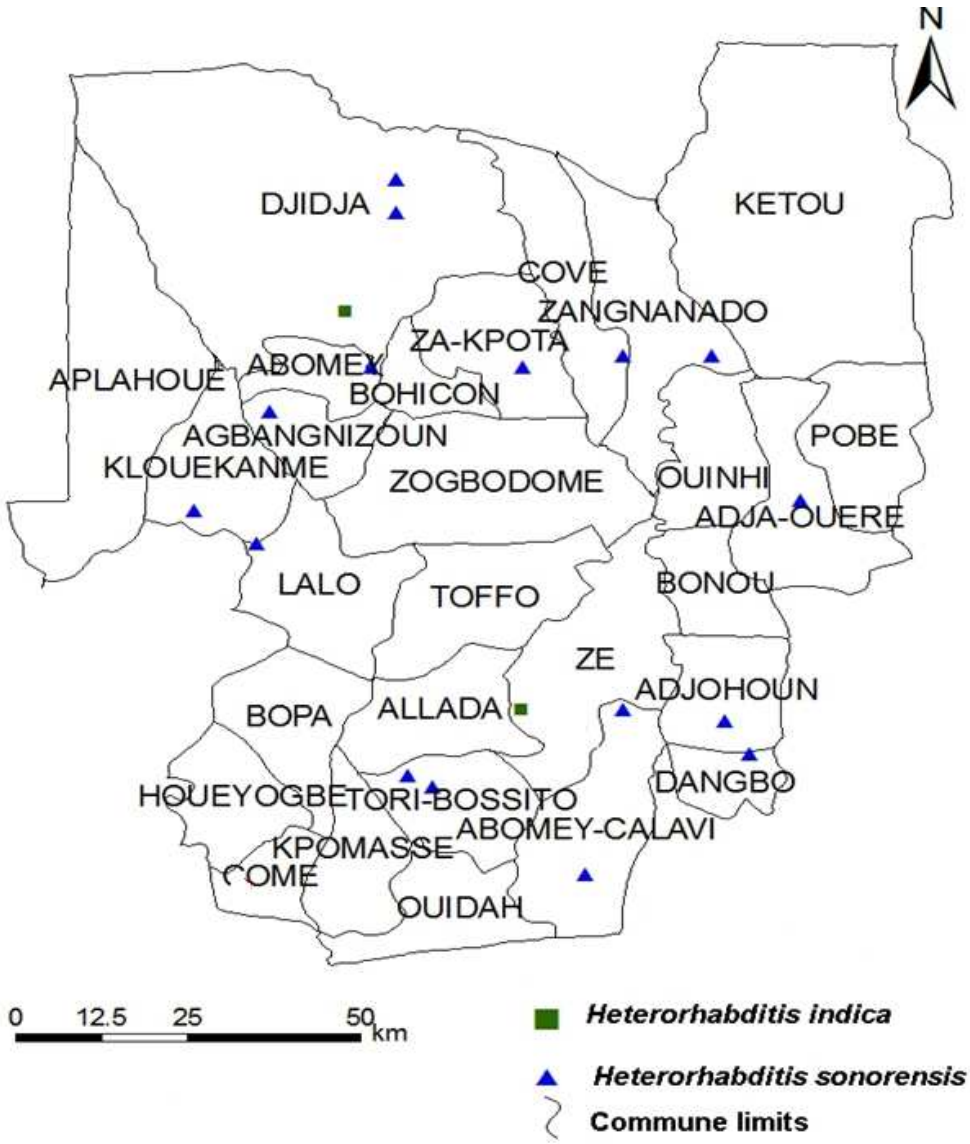

Figure 2.4 Map of south Benin showing spatial distribution of *Heterorhabditis* spp. Each symbol often represents more than one site.

1249 **Table 2.3** Distribution of heterorhabditids in south Benin at ranges of different environmental
1250 variables.

Categories (total samples)	Recovery frequency (%)[a]	Positive samples	
		No[b]	Percentage[c]
Latitude			
[1°39 - 2°00] (74)	5.4	4 (4-0)	12.5
[2°00 - 2°38] (206)	13.6	28 (25-3)	87.5
Longitude			
[6°24 - 7°00] (171)	9.4	16 (15-1)	50
[7°00 - 7°30] (109)	14.7	16 (14-2)	50
Altitude (m)			
[7 - 50] (146)	11.6	17 (15-2)	53.1
[50 - 150] (115)	12.2	14 (13-1)	43.8
[150 - 252] (19)	5.3	1 (1-0)	3.1
Habitat			
Citrus orchards (167)	9.6	16 (15-1)	50
Fruit fields (56)	17.9	10 (8-2)	31.3
Woodland (44)	13.6	6 (6-0)	18.8
Crop fields (13)	0	0	0
pH			
[4 - 5] (24)	4.2	1 (1-0)	3.1
[5 - 6] (127)	9.5	12 (11-1)	37.5
[6 - 7] (106)	16	17 (15-2)	53.1
[7 - 8] (23)	8.7	2	6.3
Silt content % (0.002-0.05mm)			
[0 - 10] (214)	12.2	26 (23-3)	81.3
[10 - 20] (36)	11.1	4 (4-0)	12.5
[20 - 30] (21)	4.8	1 (1-0)	3.1
[30 - 40] (6)	16.7	1 (1-0)	3.1
[40 - 50] (3)	0	0	0.00
Sand content % (0.05 - 2mm)			
[30 - 40] (8)	0	0	0.00
[40 - 50] (12)	0	0	0.00
[50 - 60] (13)	15.4	2 (2-0)	6.3
[60 - 70] (29)	13.8	4 (4-0)	12.5
[70 - 80] (82)	13.4	11 (11-0)	34.4
[80 - 90] (136)	11	15 (12-3)	46.9
Clay content % (<0.002mm)			
[4 - 10] (37)	13.5	5 (5-0)	15.6
[10 - 20] (189)	12.2	23 (22-1)	71.9
[20 - 30] (40)	7.5	3 (1-2)	9.4
[30 - 40] (6)	16.7	1 (1-0)	3.1
[40 - 50] (8)	0	0	0
Organic matter content %			
[0 - 1] (37)	10.8	4 (4-0)	12.5
[1 - 2] (85)	5.9	5 (5-0)	15.6
[2 - 3] (94)	14.9	14 (11-3)	43.8
[3 - 4] (37)	8.1	3 (3-0)	9.4
[4 - 5] (15)	26.7	4 (4-0)	12.5
[5 - 6] (12)	16.7	2 (2-0)	6.3
Soil texture			
sand (144)	11.1	16 (15-1)	50
sandy clay (100)	13	13 (11-2)	40.6
sandy loam (23)	8.7	2 (2-0)	6.3
sandy clay loam (13)	7.7	1 (1-0)	3.1

1251 [a] Recovery frequency (number of positive samples/total number of samples).

1252 [b] Number positive samples (number of isolate of *H. sonorensis* – number of isolate of *H. indica*)

1253 [c] Percentage positive samples (number positive samples per category variable/number total positive samples).

1254

2.3.2.3 Discriminant analysis

Five variables (longitude, organic matter, silt, sand, and clay content) discriminated, as best, positive soil samples from negative soil samples (Table 2.4).

Table 2.4 Discriminant analysis of environmental variables predicting presence of entomopathogenic nematodes in soil samples (Bold values indicate variables in the model).

Parameters	Wilks' Lambda	Partial Lambda	p-level	1-R-square
Longitude	**0.961002**	**0.968678**	**0.012987**	**0.211826**
Silt	**0.950930**	**0.978939**	**0.054721**	**0.980739**
Organic matter	**0.942605**	**0.987585**	**0.181723**	**0.200838**
Sand	**0.945439**	**0.984624**	**0.120621**	**0.991339**
Clay	**0.943223**	**0.986937**	**0.166158**	**0.977789**
pH	0.927415	0.996254	0.600206	0.181189
Altitude	0.925494	0.994190	0.452738	0.328629
Habitat	0.930714	0.999798	0.972891	0.141064
Latitude	0.926365	0.995126	0.514501	0.319167

2.3.2.4 Redundancy analysis

An overall test of significance showed that the canonical relationship between EPN presence and selected environmental variables is highly significant ($p = 0.016$ after 1000 permutations; Pseudo $F = 0.115$). The RDA axis 1 explains 96.3% of the total variance of the data, which is sufficient to describe the relation between environmental variables and EPN presence. Standardized canonical coefficients for explanatory variables are given in Table 2.5. Magnitudes of these coefficients represent their relative contributions to the related axis. All variables for the canonical axis 1 have negative coefficients while coefficient of sand, silt and clay demonstrate the highest contribution of these characteristics to the canonical axis in absolute value.

When the variables and species were projected on the plane of axis 1 and 2, EPN-positive soil samples, the arrows of the variables longitude, sand, and organic matter pointed at the left

direction, whereas the arrows of the variables clay and silt pointed at the opposite direction. Arrows indicate the direction of increasing of either the values of the significant environmental variables or species presence. As a result, the presence of *H. sonorensis* and *H. indica* is correlated with increases of longitude, sand and organic matter content and a decrease of clay and silt content (Figure 2.5). However, no difference in individual ecological preferences was observed between the two species.

Table 2.5 Standardized canonical coefficients, eigenvalues and percentages of inertia from redundancy analysis.

Variables	**Axes**		
	1	**2**	**3**
Silt	-21.854	10.938	-2.916
Sand	-33.342	16.551	-4.125
Clay	-19.859	9.924	-2.410
Organic matter	-0.243	0.118	0.060
Longitude	-0.460	0.010	0.092
Eigenvalue	0.128	0.004	0.001
Constrained inertia (%)	96.254	2.802	0.944
Cumulative %	96.254	99.056	100.000

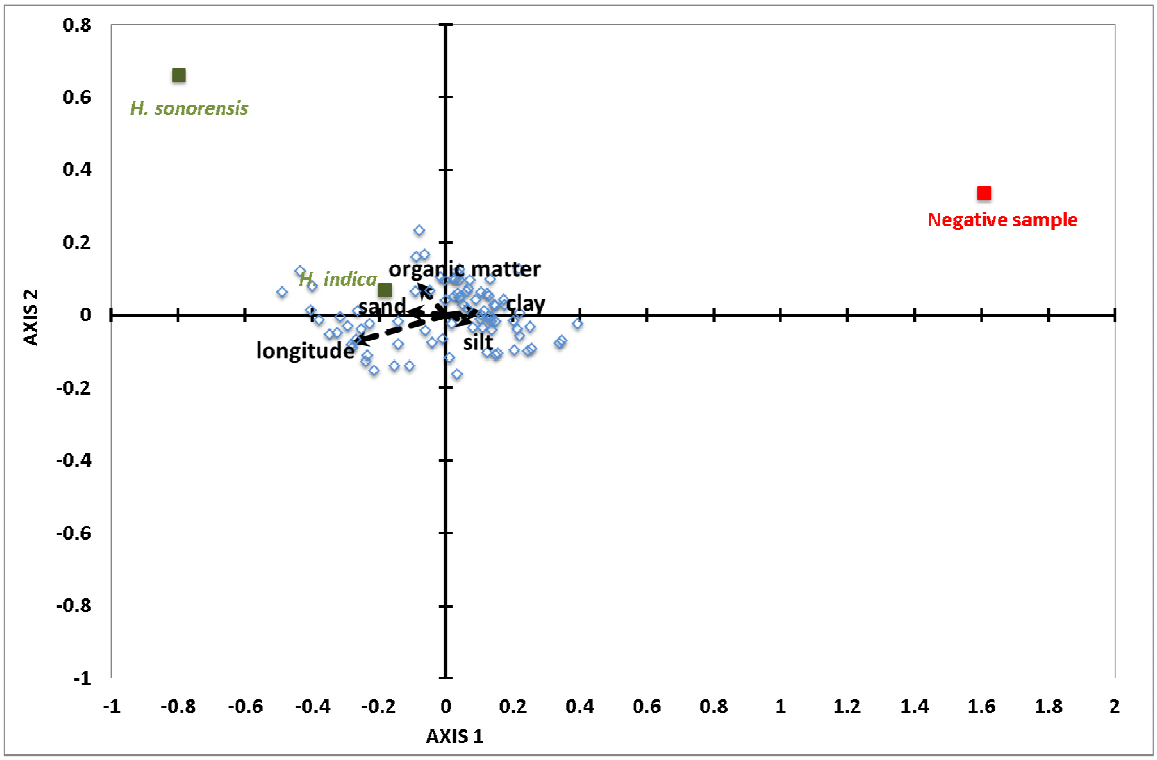

Figure 2.5 Redundancy analysis triplot showing correlation between *Heterorhabditis*-positive samples, *Heterorhabditis*-negative samples and environmental variables (longitude, silt, sand, clay and organic matter content). Dots represent often more than one sample.

2.4 DISCUSSION

The present study reports for the first time the occurrence of EPN in Benin. During the surveys conducted in 2010 and 2011, only the genus *Heterorhabditis* was recorded. This could be due to: (i) the low prevalence of *Steinernema* genus as compared with *Heterorhabditis* genus, (ii) the difference in biology of the two genera. One heterorhabditid is sufficient to multiply after invasion while at least two steinernematids (IJ to develop in male and female) must invade before reproduction can occur (Downes & Griffin, 1996), (iii) the restricted sampling; a more intensive sampling might have yielded additional species, and (iv) the use of *G. mellonella* as only baiting insect; several different hosts might have yielded additional species. As a consequence, the *Galleria*-bait method may not have allowed us to

identify all EPN present. Occasionally, the method does not detect EPN in positive samples (Spiridonov & Moens, 1999).

Out of 280 samples, 32 were EPN-positive (11.43%). Prevalence of EPN varies widely between different surveys, and ranges between 2% and 45% (Hominick, 2002). However, the recovery frequency value recorded in south Benin is within the ranges reported in African countries: Egypt (10%) (Shamseldean & Abd-Elgawad, 1994), Ethiopia (6.9%) (Mekete *et al*., 2005), Central Kenya (24%) (Waturu, 1998), and south Cameroon (10.4%) (Kanga *et al*., 2012).

This is the second record of *H. sonorensis* originally described from the Sonoran desert in Mexico (Stock *et al*., 2009) and so the first record for Africa, expanding its currently known geographic range. The presence of *H. sonorensis* in these two different parts of the world is probably due to that they both prefer a tropical climate. South Benin has a subtropical climate with two rainy seasons, whereas the Sonoran Desert has a dry tropical climate also with two rainy seasons. Moreover, in the Sonoran desert, about half of the biota is tropical in origin. The finding of *H. indica* in south Benin demonstrates its prevalence in tropical and subtropical zones (Hominick, 2002). The species was first isolated in Coimbatore, India (Poinar *et al*., 1992); later its wide distribution in the Asian continent was demonstrated by its detection in Malaysia (Mason *et al*., 1996), Japan (Yoshida *et al.*, 1998), Indonesia (Griffin *et al*, 2000); Palestine (Sansour & Iraki, 2000), Vietnam (Phan *et al*., 2001), Saudi Arabia (Saleh *et al*., 2001), and Pakistan (Anis *et al*., 2000; Shahina *et al*., 2001; Shahina & Mahreen, 2010). On the American continent, *H. indica* was reported from Cuba (Mráček *et al*., 1994), Guadeloupe, Jamaica, Dominican Republic, Martinique, Puerto Rico, and Trinidad (Constant *et al*., 1998; Fisher-Le Saux *et al*., 1998), Venezuela (Rosales & Suarez, 1998), the Virgin Islands and Florida (Stack *et al*., 2000). In Africa, it was only detected in Kenya (Waturu, 1998) and Egypt (Grenier *et al*., 1996; Stack *et al*., 2000). The species was also reported in Australia (Akhurst, 1987).

Although the majority of the soil samples were taken from citrus orchards (164/280), the principal component analysis has revealed high diversity patterns among sampled sites. The

area surveyed covered wide ranges of pH of the soil (4 - 8), silt (0 - 50%), sand (30 - 90%), clay (4 - 40%) and organic matter (0 - 6%) content, soil texture (sand, sandy loam, sandy clay loam and sandy clay), habitat (citrus orchards, fruit fields, woodland and crop fields), and altitude (7 - 252 m). However, no EPN were recovered from arable crop fields. This is in contrast to Akhurst and Brooks (1984), Hominick and Briscoe (1990) and Griffin *et al*. (1991) who observed more EPN in agricultural fields. Our observations might be biased by the survey periods, when fields were highly disturbed by farmer's practices, including use of fertilizers and pesticides.

Like many other surveys (Kung *et al*., 1990b; Griffin *et al*., 1991; Hara *et al*., 1991; Rueda *et al*., 1993; Choo *et al*., 1995; Liu & Berry, 1995; Miduturi *et al*., 1996; Hazir *et al*., 2003; Kanga *et al*., 2012) our study showed that sandy soil is the preferred soil texture for EPN. Earlier it was shown that both mobility and survival of EPN are favoured in soils with a high sand content, whereas soils with high clay content restrict nematode movements (Molyneux & Bedding, 1984; Kung *et al*., 1990b). In terms of pH-tolerance, *H. indica* was only isolated from slightly acidic (pH = 5.4 - 6.4) soils; *H. sonorensis* was isolated from acidic (pH = 5) to neutral (pH = 7.1) soils. This agrees with other studies in which the pH of *Heterorhabditis*-positive soil samples varied from 4.3 to 7.0 (Canhilal & Carner, 2006), and between 4.6 and 8 (Hara *et al*., 1991; Griffin *et al*., 1994). In most agro-ecosystems the pH (range: 4 - 8) is not likely to have any significant effect on EPN presence; however, pH $\geq$ 10 is likely to be detrimental (Kung *et al*., 1990a). *Heterorhabditis indica* was isolated from soils with 2.4 - 2.9% organic matter content, whereas *H. sonorensis* was isolated from soils with a larger range (0.1 – 4.7%). Canhilal and Carner (2006) reported that the organic matter content of *Heterorhabditis*-positive soils collected in South Carolina averaged 3% (0.7 - 7.8). In heterorhabditid-positive soil samples collected in the Central Rift Valley Region of Kenya a range of 2 - 3% was reported by Mwaniki *et al.* (2008). Earlier reports suggest that *H. indica* is not restricted by vegetation type (Griffin *et al*., 2000); however, during the present survey, *H. indica* was only observed from citrus orchards and fruit production fields, whereas *H. sonorensis* was observed from citrus orchards, fruit production fields and woodland. This suggests that, the influence of agricultural activities on *H. sonorensis* seems to be less than on

H. indica in our survey. Moreover, the differences in the distribution of these two species could be the result of the availability or differences in the distribution of suitable host insects.

Discriminant analysis identified longitude, organic matter content and soil texture (silt, sand and clay content) to be the most important abiotic factors determining the occurrence of EPN in soil from south Benin. Using these parameters, redundancy analysis results revealed *H. sonorensis and H. indica* to prefer soil with high sand or organic matter content located in the more eastern longitude; in other words, soil with decreasing silt and clay content. However, no significant difference was observed in ecological trends of EPN species taken individually. Soil texture is essential for the existence of some *Heterorhabditis* species and accordingly plays an important role in the EPN dispersal and persistence (Georgis & Poinar, 1983; Kung *et al*., 1990b; Sturhan, 1999).

The crossbreeding test between *H. indica* Ayogbe1 and *H. indica* LN2 yielded fertile progeny. As a consequence we concluded this isolate to be conspecific with *H. indica*. However in term of *H. sonorensis* isolates, although no progeny was obtained from the crossing between tested *H. sonorensis* isolates and *H. taysearae*, the closest isolate, crosses with a type specimen would have provided a final result on the identification of the species found.

This was the first attempt to study the occurrence and diversity of EPN in Benin. In comparison with other studies the diversity of nematodes yielded from this is rather low with no record of *Steinernema*. However, a survey conducted later has revealed, based on molecular analysis, the occurrence of 4 new undescribed *Steinernema* species in the northern part of the country. Morphological/morphometric and crossbreeding tests are still needed to confirm these newly collected nematodes identities.

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Characterization of biocontrol traits of heterorhabditid entomopathogenic nematode isolates from South Benin targeting the termite pest *Macrotermes bellicosus*

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ABSTRACT

Twenty-nine Beninese isolates of *Heterorhabditis sonorensis* and one local isolate of *H. indica* were screened in bioassays for their pathogenicity against *Macrotermes bellicosus* and for their tolerance to heat (40°C), desiccation (25% glycerine) and hypoxic conditions. The bioassays showed significant differences among isolates for all tested traits. Most of the isolates (73%) killed more than 80% of the insects. The greatest survival of infective juveniles to heat (8 h), desiccation (8 h), and hypoxia (72 h) was observed with the *H. sonorensis* isolates Kassehlo (72.8%), Setto1 (72.5%), and Kissamey (81.5%, respectively). Hierarchical cluster analysis identified six clusters. One of the clusters grouped three isolates of *H. sonorensis* (Zoundomey, Akohoun and Kassehlo) that scored well for all traits. These latter isolates were similarly effective by suppressing within five days a population of *M. bellicosus* naturally imprisoned in 1000-cm^3 containers and inoculated with a single two-weeks old EPN-infected *Galleria* larva.

3.1 INTRODUCTION

Entomopathogenic nematodes (EPN) of the genera *Steinernema* (Panagrolaimomorpha: Steinernematidae) and *Heterorhabditis* (Rhabditida: Heterorhabditidae) are effective biocontrol agents of a variety of economically important insect pest (Grewal *et al*., 2005). Species of the genera *Steinernema* and *Heterorhabditis* live symbiotically with bacteria of the genera *Xenorhabdus* and *Photorhabdus* (Enterobacteriacae), respectively (Ciche *et al*., 2006). Upon its entry into the host's haemocoel, the infective juvenile (IJ) of the nematode releases its symbiotic bacteria, which proliferate, kill the insect within 2 days and serve as a food source for the nematodes (Dowds & Peters, 2002). The IJ is the only free-living stage occurring naturally in the soil and is faced with a wide array of environmental conditions such as soil texture, soil pH, moisture, temperature and aeration (Glazer, 2002). Indigenous EPN are best adapted to local abiotic conditions and consequently provide the greatest chances as biological control agent (Grewal *et al*., 1994).

To obtain effective native EPN isolates for the biological control of a local termite pest *Macrotermes bellicosus*, surveys were conducted in South Benin. They yielded 32 isolates, all belonging to the genus *Heterorhabditis*; 29 of them were conspecific with *H. sonorensis* Stock, Rivera-Orduno & Flores-Lara, 2009 and the remaining three were conspecific with *H. indica* Poinar, Karunakar and David, 1992 (Chapter 2). *Heterorhabditis indica* is a worldwide distributed species (Hominick, 2002) and commercialized as a control agent for various insect pests such as the fall armyworm *Spodoptera frugiperda* (Negrisoli *et al*., 2010), the Indian meal moth *Plodia interpunctella* (Mbata *et al*., 2010), and the root weevil *Diaprepes abbreviatus* (Shapiro-Ilan *et al.,* 2010). However, little information is known about the beneficial traits of *H. sonorensis*, which was recorded in Benin for the second time (Chapter 2) after its description from the Sonoran desert in Mexico (Stock *et al*., 2009).

When selecting a particular strain for biological control, its virulence towards the target insect species and its tolerance to environmental extremes are criteria of paramount importance. In view of the hot temperatures which may exceptionally reach 35-40°C in southern Benin, and the local predominance of vertisol and hydromorphic soils (Faure & Volkoff, 1998), we

hypothesized that indigenous EPN isolates with a good ability to withstand heat, desiccation and low oxygen levels may be promising candidates to be used in biocontrol programmes.

I here report the results of the screening of these Beninese isolates for their tolerance to heat, desiccation and hypoxia, and the effect of these environmental extremes on their pathogenicity in laboratory tests. The screening was completed with observations on the virulence against *M. bellicosus* in semi-field conditions.

3.2 MATERIALS AND METHODS

3.2.1 Nematodes and insects

All isolates (Table 3.1) were multiplied in last instar larvae of *Galleria mellonella* (0.22 ± 0.018 g), which were incubated at 27°C for 72 h. Cadavers were transferred to modified White traps (Kaya & Stock, 1997) to collect IJ, which were stored at 13-15°C for no longer than 3 weeks. Before use, they were acclimated at room temperature (25°C) for 2 h and their viability (on the basis of movement) was checked under a stereomicroscope. The concentrations of IJ required for the bioassays were adjusted by volumetric dilutions in distilled water (Navon & Ascher, 2000).

Workers of *M. bellicosus* (0.033 ± 0.009 g) were collected from a citrus field and transferred to 1000-ml plastic boxes containing small pieces of moist paper (food) and wet sand collected from termite nests. The boxes were maintained in the dark at 25°C and 75-80% RH for 24 h before being used. Only very active termites were selected for use.

3.2.2 Pathogenicity of isolates to *Macrotermes bellicosus*

Eppendorf tubes (2 ml) filled with 1 ml pasteurized air-dried sand adjusted to 10% moisture (w/w) were amended with 50 IJ of one of the 30 isolates suspended in 60 µl distilled water. One worker of *M. bellicosus* was transferred to the tube, which was then closed with its perforated lid. The control tubes received 60 µl distilled water without nematodes. Termites

were provided with a thin dried straw as food source. The tubes were arranged in a completely randomized block design and kept at room temperature (25°C). Insect mortality was recorded after 48 h. Four replicates were prepared for each isolate and each replicate consisted of 12 Eppendorf tubes. The experiment was repeated twice using different batches of nematodes. Dead insects were dissected 24 h after their death to confirm nematode infection.

3.2.3 Environmental tolerance

Heat tolerance was assessed using a method modified from Shapiro *et al*. (1996). Approximately, 2,000 IJ in 1 ml water were transferred to a 20-ml assay tube. The tube was sealed with Parafilm M and incubated in the dark at 40°C for 2, 4, 6 or 8 h whilst being shaken at 70 rpm. After each period, 9 ml distilled water was added to the tube kept for an additional 24 h at room temperature (25°C) to allow the nematodes to recover from the heat shock. Tubes maintained at 25°C were used as controls. The number of dead and live nematodes was estimated on three aliquots of 100 µl taken from the homogenised nematode suspension. Inactive nematodes were considered dead if they did not respond to probing. The tubes were arranged in a completely randomized block design with three replications including the 30 isolates used in the pathogenicity experiment. The experiment was performed twice using different batches of nematodes.

Desiccation tolerance was evaluated by dehydrating IJ of the isolates in a glycerol solution (Glazer & Salame, 2000). Briefly, 0.5 ml of water containing 1000 IJ was first transferred in a 20-ml assay tube. Afterwards, 0.5 ml of 50% (v/v) glycerol solution was pipetted into the tube to obtain a final glycerol concentration of 25% (v/v). The tube was incubated in the dark at 25°C and 75–85% RH, for 6, 8, 10 or 12 h, with gentle shaking (70 rpm). After each period, the IJ were rehydrated by adding 15 ml distilled water. Tubes with the same concentration of IJ and kept at the same temperature during the same time were used as control for each isolate. The tubes were arranged in a completely randomized block design with four replicates

per treatment. Nematode survival was assessed 24 h after rehydration at 25°C as described above. The experiment was performed twice using different batches of IJ.

Hypoxia tolerance was evaluated as described by Somasekhar *et al*. (2002). Five thousand IJ suspended in 0.5 ml distilled water were transferred to a 0.5-ml Eppendorf tube. The tube was closed and kept in the dark at 25°C for 24 or 72 h. After treatment, the nematodes were suspended into 15 ml water in a Petri dish and kept at 25°C in the dark for an additional 24 h. Open Eppendorf tubes constantly kept at 25°C in the dark were used as controls. The number of dead and live nematodes was estimated as described in the heat tolerance experiment. The tubes were arranged in a completely randomized block design with four replications per treatment. The experiment was performed twice using different batches of nematodes including all 30 isolates.

Effect of environmental extremes on nematode pathogenicity. The effect of heat, desiccation and hypoxia on nematode pathogenicity was screened using IJ from the experiments described above. The screening was restricted to IJ of those isolates whom survival to heat (8 h exposure), desiccation (8 h exposure) or hypoxia (72 h exposure) was greater than 50 % (Table 3.2). Before being used, treated IJ were kept in dark at 25°C for an additional 48 h after stress treatments. Pathogenicity was evaluated by assessing the mortality caused by treated IJ to last instar larvae of *G. mellonella* as described above. Fifty IJ suspended in 60 µl sterile distilled water were transferred to individual 2-ml Eppendorf tubes containing 1 ml of pasteurized air-dried sand adjusted to 10% moisture (w/w). Thereafter, a single last instar *G. mellonella* was added. For the control, non-treated IJ were used. The tubes were kept in the dark at room temperature (25°C). Insect mortality was recorded 72 h after inoculation. Dead insects were dissected to ascertain the presence of EPN. Four replicates were used per isolate with eight Eppendorf tubes per replicate. The experiment was performed twice.

3.2.4 Semi-field evaluation of the biological control by *Macrotermes bellicosus*

Because of their ability to tolerate heat, desiccation and hypoxic conditions in the previous experiments, three isolates of *H. sonorensis* (Zoundomey, Akohoun and Kassehlo) were

selected for a semi-field evaluation during the rainy season (May-July 2013). For this purpose, 3 termite nests (diam. 400-500 cm at the base; 120-175 cm high) were selected at the origin of the isolates; they were partially broken at their base at four equidistant points to make a hole (10 cm diam. and 15 cm deep). The broken parts were drenched with 1 litre sterile water before a plastic container (1000 cm^3) was inserted into each broken point. The range of soil temperature and humidity at 5-10 cm depth inside the termite nests was 26-31°C and 12-20 % moisture (W/W), respectively. The soil texture was sandy clay and free of EPN.

In three days time, local termites had rebuilt the broken parts and colonised naturally the interior of the containers. At day four, either 5,000 (treatment 1) or 10,000 (treatment 2) IJ suspended in 5 ml sterile water, or a single 2-week old EPN-infected *G. mellonella* larva along with 5 ml sterile water (treatment 3) were applied to the top of the soil in the containers. A single EPN-infected *G. mellonella* larva held *ca.* 200,000 IJ. The control received only 5 ml sterile water. The containers were covered with their lids with 10 perforations (ca. 0.6 mm diam.) to allow air exchange. The containers were left in the nest for five days. After that period, they were removed and dead and live termites were counted. For each treatment in each replicate, 20 dead termites were randomly selected and dissected in Ringer's solution under a dissecting microscope to confirm parasitism by EPN. There were 9 termite nests, each holding 4 containers (i.e. 1 for each of the 3 treatments and 1 for the control). Each treatment was replicated three times and the experiment was repeated twice in the same conditions with different batches of nematodes and different termite nests.

3.2.5 Statistical analysis

In pathogenicity experiments, bioassays that showed control mortality were discarded and further repeated. Only pathogenicity bioassays that showed no mortality in control were considered. No IJ mortality was observed in the control treatment for the environmental tolerance bioassays. Likewise, no *G. mellonella larva* mortality was observed in the control treatment for the bioassays on the effect of environmental extremes on nematode pathogenicity. In semi-field experiments, control mortality did not exceed 10%, then insect

mortality data were corrected according to Abbott (1925). Because data from repeated bioassays were not significantly different they were pooled for analysis. To stabilize the variance of means, mortality percentages were arcsine transformed and subjected to General Linear Model (GLM) analysis performed with SAS 9.2. Student-Newman-Keuls (SNK) test at $P < 0.05$ was carried out to assess differences among nematode isolates. Non-transformed means are presented in figures. Multivariate and correlation analysis were run using STATISTICA 7.1 to investigate diversity between isolates regarding their ability to tolerate unfavourable environmental stresses. Thus studied variables pathogenicity to *M. bellicosus* and tolerance to heat (8 h exposure), desiccation (8 h exposure) and hypoxia (72 h exposure) were used in Hierarchical Cluster Analysis (HCA) to depict nematode isolates' typology. HCA used Ward's method as linkage criterion, Euclidean distance as metric (measure of distance between pairs of observations) and values of variables were standardized with Z-transformation. The dendrogram similarity scales generated by STATISTICA ranged from zero (greater similarity) to 5 (lower similarity).

3.3 RESULTS

3.3.1 Pathogenicity of EPN isolates to *Macrotermes bellicosus*

The mortality of *M. bellicosus* was significantly different between the isolates ($F = 10.38$; df = 29, 150; $P < 0.0001$) (Table 3.1) and varied between 52.8% and 98.6%. The most pathogenic isolates (SNK's test) were *H. sonorensis* Akare and *H. sonorensis* Azohoue1 (98.6% mortality); the least pathogenic isolates were *H. sonorensis* Avokanzoun (52.8%) and *H. sonorensis* Ze4 (58.3%). The *H. indica* isolate caused 83.3% mortality to *M. bellicosus.* Seventy three percent of the EPN isolates induced more than 80 % mortality to *M. bellicosus* (Table 3.1).

Table 3.1 Mortality (% ± SEM) of *Macrotermes bellicosus* 48 h after exposure to 30 different *Heterorhabditis* EPN isolates from South Benin.

EPN species	Isolates	Accession numbers	Origin of nematode isolates (Village, Department)	Mortality to *M. bellicosus* (% ± SE)
H. sonorensis	Akare	KF723798	Oko-Akare, Plateau	98.6 ± 1.4 a
	Azohoue1	KF723808	Azohoue Azongo, Atlantique	98.6 ± 1.4 a
	Kissamey	KF723806	Kissamey, Couffo	97.2 ± 1.8 ab
	Yokon	KF723801	Yokon, Oueme	95.8 ± 1.9 abc
	Zoundomey	KF723805	Zoundomey, Couffo	95.8 ± 1.9 abc
	Hessa2	KF723803	Hessa, Oueme	94.4 ± 1.8 abc
	Ze3	KF723828	Ze, Atlantique	93.1 ± 1.4 abc
	Aliho	KF723807	Azohoue Aliho, Atlantique	93.1 ± 3.3 abc
	Azohoue2	KF723809	Azohoue Azongo, Atlantique	91.7 ± 2.2 abc
	Kemondji	KF723813	Kemondji, Zou	90.3 ± 4 abc
	Djidja1	KF723822	Djidja, Zou	88.9 ± 4.1 abcd
	Ze1	KF723827	Ze, Atlantique	87.5 ± 2.8 bcde
	Zagnanado	KF723814	Zagnanado, Zou	87.5 ± 3 bcde
	Aglali	KF723804	Aglali, Couffo	86.1 ± 1.8 bcdefg
	Djidja2	KF723823	Kassehlo, Zou	86.1 ± 4.6 abcde
	Dan	KF723825	Avokanzoun, Zou	84.7 ± 4 bcdef
	Ouere2	KF723800	Adja-Ouere, Plateau	84.7 ± 3.3 bcdefg
	Ouere1	KF723799	Adja-Ouere, Plateau	83.3 ± 4.3 bcdefg
	Hessa1	KF723802	Hessa, Oueme	83.3 ± 3 cdefg
	Kpedekpo	KF723815	Kpedekpo, Zou	83.3 ± 1.9 cdefg
	Akohoun	KF723818	Akohoun, Zou	83.3 ± 3 cdefg
	Tankpe	KF723812	Tankpe, Atlantique	72.2 ± 3.5 defgh
	Setto3	KF723821	Setto, Zou	70.8 ± 3.6 efgh
	Kpanroun	KF723810	Kpanrou, Atlantique	69.4 ± 2.8 efgh
	Setto1	KF723819	Setto, Zou	68.1 ± 2.6 efgh
	Setto2	KF723820	Setto, Zou	63.9 ± 1.8 efgh
	Kassehlo	KF723824	Dan, Zou	62.5 ± 1.9 gh
	Ze4	KF723829	Ayogbe, Zou	58.3 ± 4.8 h
	Avokanzoun	KF723826	Ze, Atlantique	52.8 ± 5.1 h
H. indica	Ayogbe1	KF723816	Djidja, Zou	83.3 ± 5.7 bcde

Means (% ± SEM) within a column followed by the same letter are not significantly different (SNK's test, $P < 0.05$). SE=Standard Error of Mean.

3.3.2 Environmental tolerance

Heat tolerance. The survival of IJ after exposure to 40°C was significantly affected by the isolates (F = 228.91; df = 29, 600; $P < 0.0001$) and exposure times (F = 1339.08, df = 3, 600; $P < 0.0001$). The interaction isolates × exposure times was also significant (F= 18.95; df = 87, 600; $P < 0.0001$). No mortality was observed in the control treatments. After 2 h at 40°C, largest survival was observed for 15 isolates of *H. sonorensis* (80.6-98.9 %); isolates Ze3, Setto1 and Dan of *H. sonorensis* were most sensitive to the treatment (3.6-12.5 % IJ survival) (Table 3.2). After 4 h, survival of IJ was largest for 6 isolates of *H. sonorensis* (88.9-97.5 %); lowest survival was observed with 12 isolates of *H. sonorensis* (0-5.8 % IJ survival) along with the *H. indica* isolate (1.9 % IJ survival). When increasing exposure time to 6 h, largest survival of IJ was observed for five isolates of *H. sonorensis*, (*viz*. Zoundomey, Zagnanado, Kpedekpo, Akohoun and Kassehlo) (78.9-89.7 %); the number of isolates with the lowest survival increased up to 17 *H. sonorensis* isolates (0-6.4 % IJ survival) and the *H. indica* isolate (0.3 % IJ survival). Exposing IJ to 40°C for 8 h yielded the best survival for six *H. sonorensis* isolates (Zoundomey, Zagnanado, Kpedekpo, Akohoun, Djidja2 and Kassehlo) showing survival greater than 50%. They all originated from the department of Zou, except isolate Zoundomey, which is from the department of Couffo. *Heterorhabditis indica* (Ayogbe1) showed poor heat tolerance. A significant and negative correlation was observed between survival of IJ after heat treatment and time of exposure (r = -0.5235; $P < 0.0001$). General responses of survival of IJ (Y) as a function of exposition time (X) were expressed as Y = 80.121- 0.5235 * X.

Desiccation tolerance. The survival of IJ after desiccation differed significantly among the isolates (F = 50.56; df = 29, 630; $P < 0.0001$) and exposure times (F = 641.8; df = 2, 630; $P < 0.0001$). The interaction isolates × exposure times was also significant (F= 3.10; df = 58, 630; $P < 0.0001$). No mortality was observed in control treatments. Survival of IJ after 6, 8 and 10 h exposure ranged between 35.6 and 82.3 %, 0 and 72.5 % and 0 and 54 %, respectively (Table 3.2). No isolate withstood desiccation in 25 % glycerol for 12 h. The majority of the isolates survived desiccation very poorly during 10 h; only two isolates of *H. sonorensis* (Kpanroun and Setto1) survived with more than 50% of IJ. When the exposure was reduced to

8 h, IJ of 7 more isolates (Zoundomey, Azohoue1, Azohoue2, Kemondji, Akohoun, Kassehlo and Dan) exhibited survival between 53.3 and 68.3 %). They are all originating from three departments (Couffo, Atlantique and Zou). *Heterorhabditis indica* (Ayogbe1) survived desiccation badly (36.1 and 0.8 % IJ survival after 6 and 8 h, respectively). A highly significant and negative correlation was observed between survival of IJ after desiccation treatment and time of exposure ($r = -0.7244$; $P < 0.001$). General responses of survival of IJ (Y) as a function of exposure time (X) were expressed as $Y = 102.18 - 8.702 * X$.

Hypoxia tolerance. The survival of IJ after hypoxia treatment differed significantly among isolates ($F = 38.95$; df = 29, 420; $P < 0.0001$) and exposure times ($F = 2793.13$; df = 1, 420; $P < 0.0001$). The interaction isolates × exposure times was also significant ($F = 8.20$; df = 29, 420; $P < 0.0001$). No IJ were found dead in the control treatments. After exposure to anoxic conditions for 24 and 72 h, survival of IJ ranged between 85.9 and 96.9 %, and 33.2 and 81.5 %, respectively (Table 3.2). All isolates survived well 24 h exposure; survival was above 85 %. After 72 h, the highest survival of IJ was observed with *H. sonorensis* Kissamey (81.5 %); *H. indica* (Ayogbe1) recorded a significantly lower survival (60.8 %). Correlation between survival of IJ and exposure time was negative and highly significant ($r = -0.8062$; $P < 0.0001$). General responses of survival of IJ (Y) as a function of exposure times (X) were expressed as $Y = 107.69 - 0.6571 * X$.

Table 3.2 Mean survival (% ± SEM) of infective juveniles of 29 *Heterorhabditis sonorensis* isolates and one *Heterorhabditis indica* isolate from south Benin after heat treatment (at 40°C, for 2, 4, 6 and 8 h); desiccation treatment (25% glycerol for 6, 8 and 10 h at 25°C and 75-85 % RH) and hypoxia (anoxic conditions for 24 and 72 h at 25°C).

EPN species	Isolates	Heat (40°C)				Desiccation (25% glycerol)				Hypoxia	
		2 h	4 h	6 h	8 h	6 h	8 h	10 h	12 h	24 h	72 h
H. sonorensis	Akare	76.9±5.3 cdefg	22.6±4.1 g	10.6±2.6 f	2.9±0.6 cd	53.3±6.1 efgh	27.5±9.4 def	10.0±6.5 de	0 a	96.3±0.4 ab	77.1±2.6 abcd
	Ouere1	65.8±6.2 efgh	2.8±1.3 hij	0.0 g	0.0 d	46.3±4 fgh	16.7±6.2 efg	0.0 e	0 a	94.9±1.5 abcde	76.6±5.4 abcd
	Ouere2	89.7±2.7 abcde	3.6±2.1 hij	0.0 g	0.0 d	36.6±0.4 h	1.7±0.6 ghi	0.0 e	0 a	92.8±0.7 defgh	67.3±3.4 bcdefg
	Yokon	32.2±4 ij	0.0 j	0.0 g	0.0 d	37.7±1 h	2.3±1.5 ghi	0.0 e	0 a	89.0±0.4 ijk	52.9±1.8 hijk
	Hessa1	84.4±4.4 abcdef	0.8±0.6 ij	0.0 g	0.0 d	54.9±6.6 defg	30.0±10.3 de	13.0±8.5 de	0 a	87.2±0.6 jk	39.5±3.1 kl
	Hessa2	94.9±2.7 abc	77.1±7 cde	4.3±2.1 g	0.0 d	63.0±1.3 bcde	42.5±2.1 bcd	2.0±0.8 e	0 a	96.2±0.6 ab	80.7±2.7 abc
	Aglali	75.8±5.4 cdefg	12.2±2.7 gh	0.0 g	0.0 d	44.2±2.1 gh	11.3±3.2 efgh	0.0 e	0 a	86.3±1.3 jk	45.1±5.3 jkl
	Zoundomey	94.3±1.8 abc	93.3±2.8 ab	86.7±3.5 a	53.3±2.7 b	75.3±4.1 abc	61.7±6.4 abc	31.5±11.2 abcd	0 a	92.5±0.4 efgh	68.7±2 bcdefg
	Kissamey	78.1±8.6 bcdef	21.9±4.9 g	18.1±2.3 e	5.6±2.1 cd	37.2±0.4 h	0.8±0.5 hi	0.0 e	0 a	94.3±0.5 bcdefg	81.5±1.5 a
	Aliho	80.6±6.2 abcdef	8.9±2.9 hi	0.0 g	0.0 d	36.1±0.4 h	0.8±0.5 hi	0.0 e	0 a	94.9±1 abcdef	77.3±4.1 abcd
	Azohoue1	61.4±3.4 fgh	0.0 j	0.0 g	0.0 d	69.9±3.2 abcd	53.3±5 abc	20.0±7.6 bcde	0 a	96.9±0.7 a	43.7±3.1 jkl
	Azohoue2	84.7±5.6 abcde	5.8±1.7 hij	1.1±0.8 g	0.0 d	73.7±7.6 abc	53.3±11.8 abc	43.5±10.6 abc	0 a	93.8±1 bcdefg	72.3±4.4 bcde
	Kpanroun	25.6±2.7 jk	0.3±0.3 j	0.0 g	0.0 d	79.6±6.9 a	68.3±10.8 ab	54.0±12.5 a	0 a	88.8±1.3 ijk	43.7±6 jkl
	Tankpe	51.9±9 ghi	0.3±0.3 j	0.0 g	0.0 d	39.9±1.6 gh	6.7±2.4 fghi	0.0 e	0 a	93.7±0.6 bcdefg	71.6±2.6 bcde
	Kemondji	91.9±4.1 abcd	85.0±2.6 bcd	61.9±1.5 b	14.4±4.9 c	73.2±1.5 abc	58.3±2.3 abc	25.0±4.1 abcd	0 a	86.2±0.7 jk	35.1±3.2 l
	Zagnanado	90.8±2.7 abcd	90.3±4.3 abc	89.7±1.8 a	59.4±5.4 ab	36.6±0.7 h	1.7±1.1 hi	0.0 e	0 a	86.1±0.2 jk	38.3±1.1 kl
	Kpedekpo	97.8±1 ab	91.1±2.4 abcd	78.9±8.3 a	64.2±8.0 ab	35.6±0.1 h	0.0 i	0.0 e	0 a	93.4±0.7 cdefg	70.8±2.9 bcdef
	Akohoun	98.9±0.6 a	97.5±1.3 a	83.9±2.2 a	71.9±5.9 a	76.9±4.5 ab	64.2±7 abc	40.5±1.3 abc	0 a	92.3±0.5 fghi	65.7±2.2 defghi
	Setto1	4.7±1.7 l	0.0 j	0.0 g	0.0 d	82.3±4 a	72.5±5.4 a	50.5±9.8 a	0 a	87.6±0.8 jk	40.3±3.6 kl
	Setto2	93.3±1.9 abcd	88.9±1.5 abcd	46.7±1.7 c	8.3±2.3 cd	65.6±4 bcde	46.7±6.3 abcd	15.5±6.3 cde	0 a	89.2±0.3 ijk	52.0±1.4 ijk
	Setto3	96.9±1.5 ab	77.2±5.3 de	21.1±2.1 e	2.8±1.3 d	42.5±1.6 gh	10.8±2.4 efgh	0.0 e	0 a	93.5±1.1 bcdefg	71.0±4.9 bcde
	Djidja1	46.1±3.8 hij	18.3±9.4 gh	6.4±4.3 g	0.0 d	36.6±6.4 h	1.7±0.6 ghi	0.0 e	0 a	90.0±0.5 hij	55.7±2.3 ghij
	Djidja2	67.9±9 defg	64.4±6.8 e	61.4±4 b	59.9±10.9 ab	35.6±0.1 h	0.0 i	0.0 e	0 a	85.9±0.3 k	33.2±1.2 l
	Kassehlo	94.2±0.9 abcd	89.4±1.9 abcd	83.9±2.8 a	72.8±4.1 a	79.6±4.4 a	68.3±6.8 ab	43.0±12.3 ab	0 a	96.2±0.3 abc	80.7±1.5 abc
	Dan	12.5±3 kl	0.0 j	0.0 g	0.0 d	74.8±5.9 abc	60.8±11 abc	37.5±12.8 abc	0 a	94.5±0.3 bcdefg	33.2±1.4 bcde
	Avokanzoun	86.7±1.1 abcdef	40.0±4.6 f	3.9±1.3 g	0.0 d	50.6±3.1efgh	23.3±4.8 def	0.0 e	0 a	94.1±0.3 bcdefg	74.0±1.4 bcde
	Ze1	90.0±2.8 abcde	81.7±1.4 cde	35.6±2.2 d	6.7±2.8 cd	36.6±0.4 h	1.7±0.6 ghi	0.0 e	0 a	91.4±0.6 ghi	57.0±3.1 fghij
	Ze3	3.6±1 l	0.0 j	0.0 g	0.0 d	61.3±1.8 cdef	40.0±2.8 e	2.5 e	0 a	95.7±0.2 abcd	78.7±0.8 abcd
	Ze4	30.0±5.1 ijk	0.0 j	0.0 g	0.0 d	35.6±0.3 h	0.0 i	0.0 e	0 a	92.8±0.2 defgh	66.7±0 cdefgh
H. indica	Ayogbe1	75.3±7.1 cdefg	1.9±1.6 ij	0.3±0.3 g	0.0 d	36.1±0.2 h	0.8±0.5 hi	0.0 e	0 a	91.2±0.2 ghi	60.8±0.8 efghi

Means (% ± SEM) within a column followed by the same letter are not significantly different (SNK's test, $P < 0.05$). SEM = Standard Error of Mean

3.3.3 Effect of environmental extremes on nematode pathogenicity

Effect of heat. Comparison of *G. mellonella* larva mortalities caused by non-heated and heated IJ revealed significant differences (F = 281.44; df = 1, 70; $P < 0.0001$). In all cases, heat treatment reduced the pathogenicity of the IJ. Mortality of *G. mellonella* larvae exposed to non-heated IJ was not influenced by the isolate (F = 0.15; df = 5, 30; $P = 0.9772$); however, mortality was affected by the isolates when insect larvae were exposed to heat-treated IJ (F = 11.92; df = 5, 30; $P < 0.0001$). When heat-treated, IJ of the isolates *H. sonorensis* Zoundomey and Kpedekpo caused the greatest mortality (70 and 66.7%, respectively) (Figure 3.1).

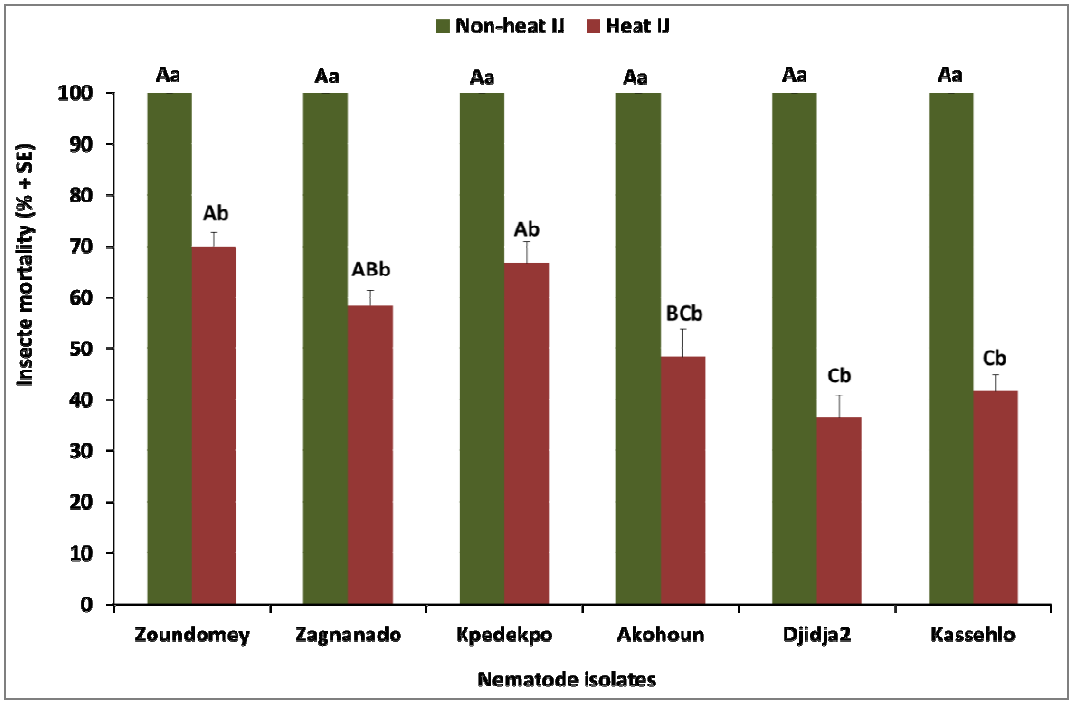

Figure 3.1 Mean percentage mortality (% ± SEM) of *Galleria mellonella* caused by non-heated (ambient temperature of 25°C) and heated (40°C) infective juveniles of six *H. sonorensis* isolates. Bars represent Standard Error of Means (SEM). Different capital letters above bars indicate statistical differences ($P < 0.05$) between percentage mortality of insect larvae caused by different nematode isolates, and different lowercase letters indicate statistical differences ($P < 0.05$) between mortality caused by heated and non-heated infective juveniles at nematode isolate level ($P < 0.05$). Isolate codes represent the same species as depicted in Table 3.1.

Effect of desiccation. Comparison of mortality of *G. mellonella* caused by non-desiccated and desiccated IJ of the same isolate revealed significant differences (F = 218.54; df = 1, 106; $P <$ 0.0001). In all cases, non-desiccated IJ were more pathogenic than desiccated ones. Mortality

caused by desiccated IJ differed significantly among nematode isolates (F = 3.18; df = 8, 45; *P* = 0.0060); however, mortality was not different between isolates when non-desiccated IJ were compared (F = 2.07; df = 8, 45; *P* = 0.0593). When comparing desiccated IJ, isolate *H. sonorensis* Setto1 (40%) and *H. sonorensis* Azohoue2 (20%) were the most and least pathogenic, respectively (Figure 3.2).

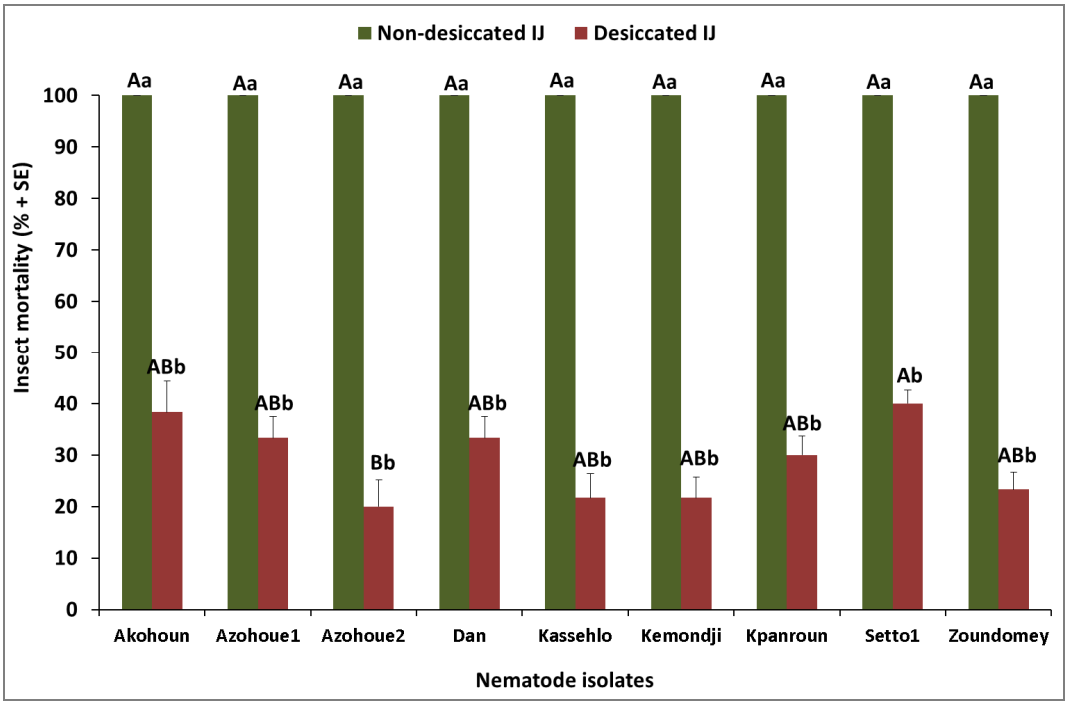

Figure 3.2 Mean percentage mortality of larvae of *Galleria mellonella* mortality (% ± SEM) caused by non-desiccated and desiccated infective juveniles of nine entomopathogenic nematode isolates. Bars represent Standard Error of Means (SEM). Different capital letters above bars indicate statistical differences among nematode isolates, and different lowercase letters indicate statistical differences between desiccated and non-desiccated infective juveniles of a given nematode isolate (SNK's test, $P < 0.05$). Isolate codes represent the same species as depicted in Table 1.

Effect of hypoxia. Comparison of mortality of *G. mellonella* caused by non-hypoxic and hypoxic IJ of the same isolate revealed significant differences (F = 392.94; df = 1, 202; *P* < 0.0001). In all cases, non-hypoxic IJ were more pathogenic than hypoxic ones. When exposing *G. mellonella* to hypoxic IJ, insect mortality was affected by the isolate (F = 5.43; df = 16, 85; *P* < 0.0001). No differences in mortality were observed between the isolates when using non-hypoxic IJ (F = 1.25; df = 16, 85; *P* = 0.2494). Within the hypoxic treated isolates, *H. sonorensis* Hessa2 (78.3%), *H. sonorensis* Zoundomey (78.3%) and *H. indica* Ayogbe1

(76.7%) caused the largest pathogenicity; *H. sonorensis* Avokanzoun (51%) was the least pathogenic (Figure 3.3).

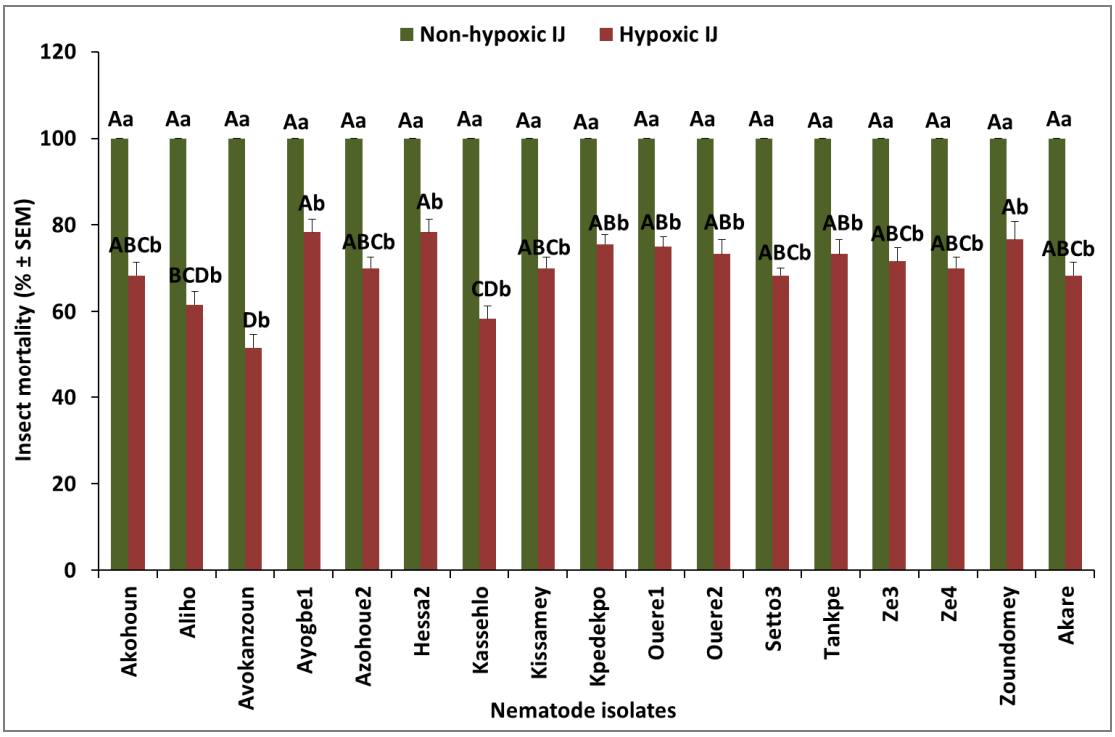

Figure 3.3 Mean percentage mortality caused to *G. mellonella* (% ± SEM) by non-hypoxic and hypoxic infective juveniles of seventeen isolates of entomopathogenic nematodes. Bars represent Standard Error of Means (SEM). Different capital letters above bars indicate statistical differences among different isolates, and different lowercase letters on bars of a given nematode isolate indicate statistical differences between mortality caused to *G. mellonella* by hypoxic and non-hypoxic IJs of the nematode isolate (SNK's test, $P < 0.05$). Isolate codes represent the same species as depicted in Table 1.

3.3.4 Hierarchical cluster analysis (HCA)

The isolates were grouped in six main clusters (Figure 3.4). ANOVA revealed significant differences among clusters in heat tolerance ($F = 55.43$; df = 5, 24; $P < 0.0001$), desiccation tolerance ($F = 65.32$; df = 5, 24; $P < 0.0001$), hypoxia tolerance ($F = 3.68$; df = 5, 24; $P < 0.0129$), as well as in pathogenicity potential to *M. bellicosus* ($F = 4.73$; df = 5, 24; $P < 0.0038$) (Table 3.3). Components of all clusters showed moderate to good pathogenicity to *M. bellicosus* with insect mortality ranging from 69.4 to 95.8 %; cluster 1 grouped the most pathogenic isolates (Table 3.3). It was composed of four isolates of *H. sonorensis* (Figure 3.4)

with poor heat tolerance (0.7 % IJ survival) but moderate desiccation tolerance (44.2 %) and good hypoxia tolerance (68.5 % IJ survival).

Table 3.3 Mean survival (% ± SEM) of infective juveniles of different clusters consisting of close *Heterorhabditis sonorensis* or *H. indica* isolates according to their ability to tolerate heat (40°C, 8 h), desiccation (25% glycerol, 8 h at 25°C and 75-85 % RH) and hypoxia (anoxic conditions for 72 h at 25°C) and their pathogenicity to *Macrotermes bellicosus* expressed by mean mortality (% ± SEM) of insect after 48 hours exposition time.

Cluster	Heat	Desiccation	Hypoxia	Pathogenicity
Cluster 1	0.7±0.7 b	44.2±6.1 b	68.5±8.4 a	95.8±1.7 a
Cluster 2	0.3±0.4 b	19.8±4.5 c	65.2±6 ab	80±4.2 b
Cluster 3	4.5±3 b	61.3±4.5 a	40.9±3.3 b	69.4±6.1 b
Cluster 4	1.5±1 b	1.2±0.3 d	64.9±3.6 ab	89.6±2.1 ab
Cluster 5	66±6.3 a	64.7±1.9 a	71.7±4.6 a	82.9±8.1 ab
Cluster 6	61.1±1.5 a	0.6±0.6 d	74.4±11.8 ab	77.8±7.7 b

Means (% ± SEM) within a column followed by the same letter are not significantly different (SKN' test, $P < 0.05$). SE=Standard Error of means

Cluster 2 included 7 isolates of *H. sonorensis* (Figure 3.4) with poor heat and desiccation tolerance (0.3 and 19.8 % IJ survival, respectively). Cluster 3 comprised 5 isolates of *H. sonorensis* (Figure 3.4) exhibiting poor heat and hypoxia tolerance (4.5 and 40.9 % IJ survival; respectively). Seven isolates of *H. sonorensis* were included in Cluster 4 along with the isolate of *H. indica* (Ayogbe1); they tolerated heat and desiccation poorly (1.5 and 1.2 % IJ survival). Cluster 5 included 3 isolates of *H. sonorensis* with good tolerance for heat (66 % IJ survival), desiccation (64.7 % IJ survival) and hypoxia (71.7 % IJ survival). Finally, like cluster 5, cluster 6 also included 3 isolates of *H. sonorensis* with good heat tolerance (61.1 % IJ survival) but poor desiccation tolerance (0.6 % IJ survival) and moderate hypoxia tolerance (74.4 % IJ survival).

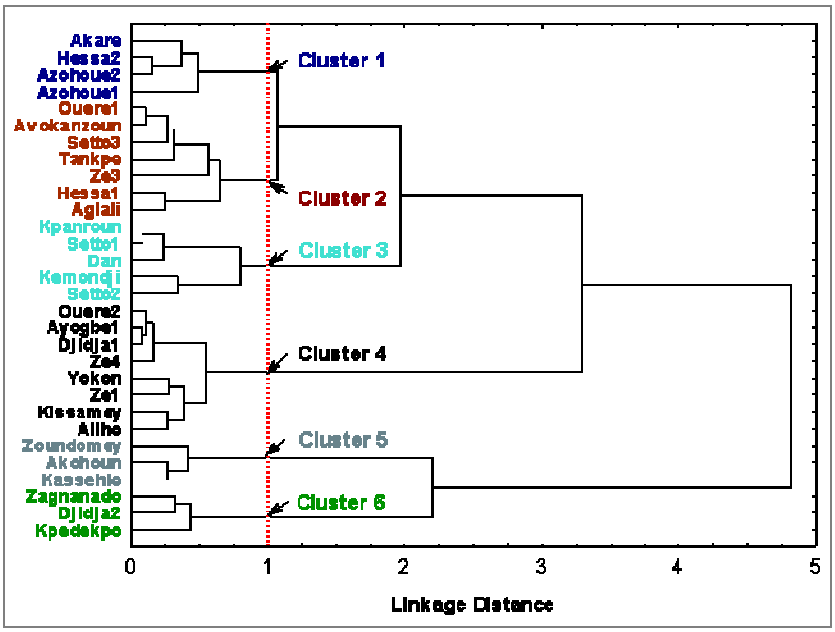

Figure 3.4 Hierarchical Tree summarising the similarities among thirty Beninean *Heterorhabditis* isolates for their pathogenicity to *Macrotermes bellicosus* and their tolerance to heat (40°C for 8 h), desiccation (25% glycerol for 8 h) and hypoxia (72 h).

3.3.5 Semi-field evaluation in the biological control of *Macrotermes bellicosus*

Generally, all nematode treatments reduced *M. bellicosus* population. The corrected mortality of *M. bellicosus* was significantly affected by the isolates ($F = 11.44$; df = 2, 63; $P < 0.0001$) and nematode treatments ($F = 282.17$; df = 2, 63; $P < 0.0001$). The interaction between both factors was also significant ($F = 3.16$; df = 4, 63; $P = 0.0197$). Accordingly, efficacy of isolates was assessed separately for a given treatment. Isolates varied greatly in terms of efficacy against *M. bellicosus* at a concentration of 5,000 IJ ($F = 4.63$; df = 2, 21; $P = 0.0216$) and 10,000 IJ ($F = 14.63$; df = 2, 21; $P = 0.0001$). However, single EPN-infected *G. mellonella* larvae induced 100% termite mortality for all isolates (Figure 3.5). Significant differences were observed among concentrations for all isolates, i.e. Zoundomey ($F = 61.14$; df = 2, 21; $P < 0.0001$), Akohoun ($F = 89.96$; df = 2, 21; $P < 0.0001$) and Kassehlo ($F = 142.48$; df = 2, 21; $P < 0.0001$). Though not significant at concentrations 5,000 IJ and 10,000 IJ, isolate Zoundomey resulted in greater mortality of *M. bellicosus* than isolates Akohoun and Kassehlo at the corresponding concentration.

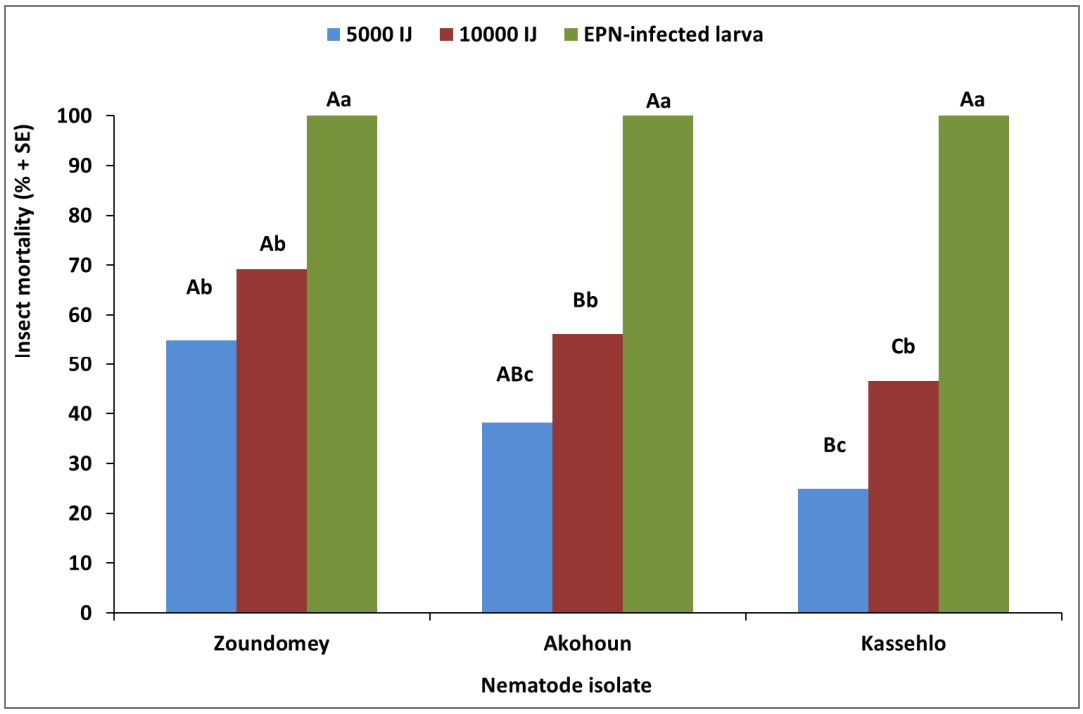

Figure 3.5 Corrected mortality (% ± SEM) of *Macrotermes bellicosus* exposed to three *Heterorhabditis sonorensis* isolates (Zoundomey, Akohoun and Kassehlo) from southern Benin at concentrations of 5,000 IJ, 10,000 IJ and emerged IJ from one two weeks old EPN-infected *Galleria* larva in 1000 cm^3-containers naturally colonised by *M. bellicosus*. Bars headed by different capital letters denote significant differences among EPN isolates in termite mortality at the same concentration. Bars headed by different small letters denote significant differences in termite mortality among nematode concentration of the same EPN isolates (SNK's test at $P < 0.05$).

3.4 DISCUSSION

Surveys for EPN often yield numerous isolates of steinernematids and/or heterorhabditids; positive samples range between 2 % and 46 % of the collected ones (Hominick, 2002). However, when these isolates are further screened for a variety of biotic (pathogenicity) and abiotic (temperature, desiccation, hypoxia) traits, the number of isolates that would justify laborious field observations, is significantly reduced (Somasekhar *et al*., 2002; Mukuka *et al*., 2010a, b; Ma *et al*., 2013). Hence, to lower the number of isolates to be tested in the field and thereby reducing the costs, it is desirable that isolates are first screened in the laboratory for a selection of qualities. To the best of our knowledge, information is lacking on both the pathogenicity of *H. sonorensis* to any pest insect, and its tolerance to environmental stress.

When searching within a collection of EPN isolates/species for candidate biocontrol isolates, the determination of their pathogenicity against the target insect is an obvious first step. Our

study clearly shows the Beninese isolates of *H. sonorensis* and *H. indica* to be pathogenic to *M. bellicosus*. Most of them (73 %) killed more than 80 % of the exposed termites. Earlier bioassays demonstrated also the efficacy of steinernematids against termites. Rouland *et al*. (1996) reported the pathogenicity of both *S. carpocapsae* and *S. kushidai* against *M. bellicosus.* Other research (Poinar & Georgis, 1989) showed that *S. carpocapsae* and *H. bacteriophora* killed various *Reticulitermes* spp. (Isoptera: Rhinotermitidae).

Next to the degree of pathogenicity against the target insect, the tolerance to environmental stresses is a paramount criterion when selecting isolates for biocontrol purposes (Glazer, 2002). Heat and desiccation are two major stress factors that reduce survival and efficiency of EPN and lower their shelf life (Strauch *et al*., 2004; Mukuka *et al*., 2010a, b). The effect of high temperature on the survival of IJ is well documented for several species of both steinernematids and heterorhabditids. Grewal *et al*. (2002) found that the survival of IJ of *H. bacteriophora* exposed to 40°C for 2 h varied between 13 and 90 % among 15 isolates, whereas Somasekhar *et al*. (2002) reported the survival to vary between 18 and 63 % among 15 isolates of *S. carpocapsae*. Fifteen of our 29 isolates of *H. sonorensis* proved to be heat tolerant with survival of IJ varying between 80.6 and 98.9% at the above mentioned temperature and exposure time. Interestingly, six of these isolates survived even an extended exposure up to 8 h (53.3-78.8 % survival).

The tolerance to desiccation, however, was considerably different between heterorhabditid isolates. Strains of *H. bacteriophora*, known as a common species in regions with continental and Mediterranean climates characterized by long and dry winters (Hominick, 2002), were reported to be able to withstand an exposure to 25% glycerol for 72 h with IJ survival varying between 25 and 90 % (Grewal *et al*., 2002). None of our isolates, however, withstood desiccation in 25 % glycerol even for a shorter period (12 h). Only when the exposure time was further reduced to 8 h, nine of the *H. sonorensis* isolates survived (> 50 % IJ). However, *H. sonorensis* was originally described from the Sonoran desert, indicating that the species must be adapted to dry and hot conditions. *H. indica* is known to be a heat and desiccation tolerant species (Mukuka *et al*., 2010a, b). However, the Beninese isolate of *H. indica* (Ayogbe1) did not survive exposure to heat (40°C for 4 h) or desiccation (25 % glycerol for 8 h). The poor desiccation tolerance observed in our EPN isolates might indicate that local isolates adapt to prevailing conditions such as association with moist soils in Benin (Shapiro-Ilan *et al*., 2005). However, our study includes only one isolate of *H. indica*; more isolates should be investigated to allow firm conclusions.

Since nematodes are aerobic organisms, hypoxic conditions can reduce their survival and longevity (Grewal *et al.*, 2011). There are large differences in the ability of EPN species and strains to withstand hypoxic conditions. While *H. bacteriophora* can withstand anoxic conditions in water at 25°C for only 4 days with survival of IJ ranging between 11 and 90 % (Grewal *et al*., 2002), *S. carpocapsae* can tolerate such conditions for up to 10 days with survival of IJ ranging between 21 and 72 % (Somasekhar *et al*., 2002). Our data revealed Beninese isolates to tolerate such conditions for 3 days with survival of IJ varying between 33.2 and 81.5 for *H. sonorensis* isolates and 60.8 % for *H. indica* Ayogbe1. This may be related to their adaptation to the lower availability of oxygen in the soils where they have been collected, since they preferred soil with high organic matter content (Chapter 2).

Environmental stress can induce loss of energy reserve of the IJ resulting in reduced attraction to the insect host (Patel *et al*., 1997), and may modify the activity of the symbiotic bacterium (Fang *et al*., 2012). However, these effects vary within isolates and species (Grewal *et al*., 1994). In our experiments, the pathogenicity of IJ against *G mellonella* larvae was significantly affected by heat (30-63.3 %), desiccation (60-78.3 %) and hypoxia (21.7-48.3 %). This suggests that the development of formulations (desiccated cadavers, capsules or baits) that provide protection to the nematodes against environmental extremes during and after application can substantially enhance nematode efficacy (Grewal, 2002). In contrast to our results, Grewal (2002) concluded that desiccation had no adverse effect on the pathogenicity of *S. feltiae*, *S. riobrave* and *S. carpocapsae*.

Selection is effective when a pool of variation is available (Strauch *et al*., 2004). Our bioassays demonstrated that different responses to environmental stress can be observed among isolates thereby corroborating previous findings (Grewal *et al*., 2002; Somasekhar *et al*., 2002; Morton & Garcia-del-Pino 2009; Grewal *et al*., 2011; Ma *et al*., 2013). The environmental characterization of EPN has been used to select better candidates for biological control. Beneficial traits were compared among isolates; isolates superior to others in most or all of the beneficial traits were selected for further investigation (Morton & García-del-Pino 2009). However, a simple visual observation of that kind of data does not allow an easy identification of superior isolates. To address this issue, we used the hierarchical approach. Hierarchical cluster analysis identified six clusters, each of them being composed of isolates that scored close to the traits tested. One of the clusters was composed of the isolates Zoundomey, Akohoun and Kassehlo of *H. sonorensis*. These three isolates scored well for all traits considered simultaneously and therefore justified their choice as best candidates for

field observations. Hierarchical Cluster Analysis organized the data into meaningful structures, and is a useful tool in the selection of EPN isolates.

Though soil dwelling EPN succeed in infecting many of soil borne insects, they are unable, under ordinary conditions, to mount so pernicious an attack that they succeed in eradicating their hosts from the common habitat (Cates, 2013). By way of contrast, in our semi-field evaluation, populations of *M. bellicosus* naturally imprisoned in a 1000 cm^3 container were effectively suppressed in five days by the selected isolates formulated as a single two-week old infected *Galleria* larva. These findings corroborate those of Cates (2013) indicating that certain EPN isolates are virulent enough to produce 100 % mortality within an inoculated termite colony; however, their virulence is limited to the inoculated point. Although, our assay arena (1000 cm^3) represents a very small part of a whole termite nest, we speculate that a higher number of EPN-infected *Galleria* larva applied at several broken parts on the termite nest may suppress the population of *M. bellicosus* inside the nest.

These studies, though conducted under controlled conditions, provide baseline data that can be useful in choosing Beninese EPN species/isolates to control *M. bellicosus*. The results from the semi-field experiment are encouraging, and experiments taking into account several approaches of EPN-infected *Galleria* larva application into termite nests are considered in my forthcoming studies.

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Effectiveness of different *Heterorhabditis* isolates from Southern Benin for biocontrol of the subterranean termite, *Macrotermes bellicosus* (Isoptera: Macrotermitinae), in laboratory trials

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ABSTRACT

The host finding ability of twenty eight *Heterorhabditis sonorensis* isolates and one *Heterorhabditis indica* isolate from southern Benin and one *Heterorhabditis indica* isolate from India were examined in vertical migration sand bioassays against workers of a target citrus termite pest, *Macrotermes bellicosus*. Thereafter, nine selected isolates were subjected to further investigations on virulence. Our results revealed that both *H. sonorensis* and *H. indica* isolates exhibit a cruiser type of search strategy and were capable to various degrees, of migrating, infecting and killing workers of *M. bellicosus* in sand columns up to 20 cm long in a period of three days. However, only three isolates of *H. sonorensis* caused 100% mortality to *M. bellicosus* at greatest depth. The exotic *H. indica* isolate (LN2) did not show high host finding ability compared to the indigenous one. Concerning virulence, differences were observed among isolates for their ability to invade workers of *M. bellicosus.* After 12 h post exposure, *H. sonorensis* from Ze (Ze3) and *H. sonorensis* from Azohoue (Azohoue2) exhibited the lowest invasion time with IT_{50} = 3.35 and 3.67 h, respectively and a higher penetration rate (11.4% and 10%, respectively) compared to the others isolates. In the concentration-mortality test, I found that, based on 95% confident limits, all *H. sonorensis* and *H. indica* isolates appeared to be equal with LC_{50} values ranging from 9 to 16 IJs/termite. Interestingly, 40 IJs/termite were enough for them to cause 80% mortality to *M. bellicosus*. Contrary to the $LC_{50,}$ the results of our studies clearly demonstrate that *M. bellicosus* exhibits a time-dependent susceptibility to the tested nematode isolates. So, the lowest LT_{50} was observed for *H. sonorensis* Ze3 (LT_{50}=23.30 h), the highest for *H. sonorensis* Yokon (34.76 h). The LT_{50} of the indigenous *H. indica* isolate was estimated to be 24.07 h. in addition, all selected isolates were able to reproduce in *M. bellicosus* workers. The highest reproduction potential in *M. bellicosus* was observed with *H. sonorensis* Yokon with 20213 IJs/termite followed by *H. sonorensis* Ze3 with 19368 IJs/termites.

All tested Beninese EPN isolates were pathogenic to the citrus termite pest *M. bellicosus* with *H. sonorensis* Ze3 being the most virulent.

4.1 INTRODUCTION

In spite of the increase of citrus production areas over the years in Southern Benin, current yields remain lower than expected due to attacks by pests and diseases from nurseries to harvest and storage (UEMOA, 2008). The losses varied according to harvest times, from 25% (in March-July) to 50% (in November-January) (UEMOA, 2008). Termites, mainly *Macrotermes bellicosus* (Smeathman), *Amitermes guineensis* (Sjöstedt), *Ancistrotermes crucifer* (Sjöstedt) and *Trinervitermes occidentalis* (Sjöstedt) are the most frequent and damaging pests found in citrus orchards in Southern Benin (ESCiP-Benin, 2011). Current termite control is done by hazardous chemicals, which adversely affect humans and environment (Tovignan *et al*., 2001; ESCiP-Benin, 2011). Therefore, the focus has increased on the application of biologically based Integrated Pest Management (IPM) systems (Lynch, 1998). However, the use of biological control agents against termites is still very limited and much more research is needed to establish which bio-control agent is effective under which parameters (Yu, 2009).

Entomopathogenic nematodes (EPN) of the families Heterorhabditidae (Rhabditida, Strongyloidea) and Steinernematidae (Rhabditida, Strongyloidoidea) are widely used in biological control for soil-dwelling stages of many insect pests being safe to most non-target organisms and to the environment (Kaya & Koppenhöfer, 2004; Ehlers, 2005). These nematodes are associated with bacterial symbionts (*Xenorhabdus* spp. for Steinernematidae and *Photorhabdus* spp. for Heterorhabditidae), which inhabit the intestine of the nematode (Burnell & Stock 2000; Boemare, 2002). It is this nematode-bacterium complex that works together to kill their insect host. The infective third stage juvenile (IJ) of the nematode is the only free living stage that searches for susceptible hosts in the soil environment. The IJ enter through natural openings (mouth, anus or spiracles) or body cuticle and release the mutualistic bacterium in the insect haemocoel (Kaya & Gaugler 1993; Campbell & Lewis, 2002). The bacterium produces toxins that cause septicaemia killing the host within 2 days while also serving as food source for the developing nematode (Poinar, 1990). When the nematodes have exhausted the food resources of the host cadaver, usually after two generations, they develop into infective larvae that leave the cadaver in search for new hosts to start a new life cycle.
Subterranean termites occupy the same soil habitat as the nematode species, but they appear not particularly susceptible to infection (Fuiji, 1975). Few studies have addressed the potential of nematodes to control termites. In an early report, Bedding and Stanfield (1981) reported that large colonies of the Australian genus *Mastotermes* could be killed using *Heterorhabditis*

sp. by injecting *Heterorhabditis* spp. directly into the infested eucalyptus trunks. Mortality exceeding 95% was recorded by Georgis *et al*. (1982) for both *Zootermopsis* sp. and *Reticulitermes* sp. within three days after laboratory exposure to *S. carpocapsae*; termites also were found to carry infection back to their colonies. Epsky and Capinera (1988) concluded that *S. carpocapsae* showed potential for control of *Reticulitermes tibialis* (Banks) in laboratory and field trials. In recent studies, the infectivity of *S. carpocapsae* (Breton), *S. riobrave* (TX), *H. bacteriophora* (HP88), and *H. indica* (Coimbatore) in *Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* (Shiraki) was investigated by Wang *et al*. (2002). In Petri dish tests, they were all effective against *C. formosanus* at a dose of 400 nematodes per termite. Yu *et al*. (2008) showed that three EPN species including *S. carpocapsae* (Mexican 33 strain), *S. feltiae* (UK76 strain) and *Heterorhabditis bacteriophora* (HP88 strain) can infect and kill desert subterranean termites *Heterotermes aureus* (Snyder) under laboratory conditions. These nematodes can also develop and reproduce in termite cadavers and emerge as infective juveniles. Furthermore, *S. riobrave* nematodes were found to cause over 75% mortality of workers of three termite species including *Gnathamitermes perplexus* (Banks), *H. aureus* and *R. flavipes* (Yu *et al*., 2006; Yu *et al*., 2010). Manzoor (2012) reported *H. bacteriophora* and *S. carpocapsae* to be effective against workers of *R. flavipes.*

Taking into consideration the characteristics of EPN, the VLIR-UOS Own Initiative 2010 project “Ecologically Sustainable Citrus Production in Benin” has initiated studies on the biodiversity of EPN and their possible use in Benin as control agents for termites. Surveys of EPN have been conducted in various habitats of southern Benin and several heterorhabditid isolates were found. The majority were identified as *Heterorhabditis sonorensis*; one isolate was identified as *H. indica* (Chapter 2). During the surveys, *M. bellicosus* was the most frequently found termite species in citrus orchards and citrus growers strongly complained about its damaging effects on citrus plants. To obtain the wood for their food, *M. bellicosus* workers make tunnels through citrus stems, weakening them and eventually causing their collapse or giving access to fungus and other diseases. Where bark is gnawed from trees, the phloem may be interrupted, causing the death of the tree. Since the insects make no openings to the outside, or do so only at an advanced stage of the invasion, the citrus growers are frequently unaware of their presence until it is too late (Femi-Ola *et al*., 2007; Kumar & Pardeshi, 2011). To control such termite pest, we hypothesise that the use of formulated insect cadavers infected with EPN as bio-pesticide could be an effective sustained solution.

The insect-pathogenic power of an EPN isolate largely depends on the capacity of IJ to migrate, penetrate and kill host. Different species of EPN employ different strategies in order to find a new host. In their foraging behaviour, EPN are categorized into ambushers, which have a 'sit-and-wait' strategy, cruisers, which actively search for their hosts, and intermediates, which show both types of foraging behaviour (Lewis, 2002). *H. indica*, for example, has a widely foraging strategy (cruiser) (Campbell & Lewis, 2002). Different strains of the same EPN species can differ in virulence and reproductive capacity (Somasekhar *et al.*, 2002).

In this chapter I report on laboratory test of comparative host finding ability of several heterorhabditid isolates in sand column assays. Virulence of nematodes against the citrus termite pests *M. bellicosus* was also investigated. The outcome will serve as basis to select the most effective EPN strain as a biocontrol agent to use in future field tests.

4.2 MATERIALS AND METHODS

4.2.1 The insects

Workers of *M. bellicosus* were collected from a citrus field. Termite nests were broken at their base, which was covered with dried straws, and left for 3-4 hours. After that period, termite infested straws were taken to the laboratory where the termites were removed and transferred to 1000 ml-plastic boxes containing small pieces of moist paper food source, and wet sand collected from the termite nest. The boxes were maintained in the dark at 25°C and 75-80% relative humidity for 24 hours before being used in the experiments. The workers weighed 0.033±0.009 g.

4.2.2 The nematodes

Nematode species/isolates involved in this study are listed in Table 4.2. All isolates were cultured in last instar larvae of the greater wax moth *Galleria mellonella* as described by Kaya and Stock (1997). Infective juveniles (IJ) were used to infect *G. mellonella* larvae, which were then incubated at 27°C for 72 h. The cadavers were transferred to White traps (White 1927); IJ were harvested and stored at 13-15°C for no longer than 3 weeks. Before use, IJ were acclimated at room temperature (25°C) for two hours and their viability on the basis of

movement was checked under a stereomicroscope. The concentrations of IJ were adjusted by volumetric dilutions in distilled water using the formula of Navon and Ascher (2000).

4.2.3 Host finding ability

In order to explore existing biological diversity within indigenous EPN isolates, and to enhance the possibility to come across outstanding ones as potential biocontrol agent, twenty-nine indigenous *Heterorhabditis* isolates (28 isolates of *H. sonorensis* and 1 one of *H. indica*) along with an exotic isolate (*H. indica* LN2) (Table 4.2) were tested for host finding ability. This latter isolate was added for comparison purpose with the new found *H. indica* isolate from Benin. Nematode migration was examined in sand column assays. Therefore, PVC tubes (4 cm diameter) of different lengths (5cm, 10cm, 15cm and 20cm) were filled with sterilized sand previously sifted over a 2-mm mesh sieve and adjusted to 10% moisture (w/w). Both ends of the tubes were plugged to avoid moisture loss. One *M. bellicosus* worker was enclosed at one of end of the tube before the tubes were kept at 25°C for 24 h for allowing insects to settle. A Mosquito net prevented the insect from moving upwards, and termites were provided with a thin dried straw as food source. Thereafter, 1000 µl water containing 1000 IJ was pipetted at the tube end opposite to the insect. The control tubes received only 1000 µl of distilled water. Upon nematode inoculation the tubes were kept vertically (with the insect at the bottom of the column) in dark at 25°C. After 72 h of incubation, insect mortality was recorded. At the same time, dead insects were rinsed and incubated for 24h at 25°C in a Petri dish lined with moist filter paper. The insects were then dissected in Ringer's solution under a stereo-microscope to ascertain that mortality resulted from nematode infection. Termite mortality and the number of infecting nematodes in each termite were recorded. The treatments were replicated 15 times and the experiment was repeated twice in the same condition with different batches of nematodes.

4.2.4 Virulence

Based on the results of the host finding ability experiments, eight *H. sonorensis* isolates (Yokon, Hessa2, Aglali, Zoundomey, Azohoue2, Kemondji, Djidja1 and Ze3) and one *H. indica* isolate (Ayogbe1) were selected for studies on the interaction between the nematodes and the termite (Table 4.2).

4.2.4.1 Invasion time

An exposure-time-assay was performed to determine the time needed by the isolates to infect a termite worker. Single worker of *M. bellicosus* was exposed during 2, 4, 6, 8, 10 and 12 h to IJ of each of the selected nematode isolates. The tests were done in sterile 9 cm-diameter Petri dishes padded with one filter paper disk (Whatman N°1). Two hundred newly harvested IJ were sprinkled onto the Petri dishes in a volume of 800 µl distilled water. The control treatment received water only. After adding IJ, the dishes were kept for 30 min at 25°C in the dark to allow the nematodes to scatter and settle down. Thereafter, one termite worker was added per Petri dish. Termites were provided with a thin dried straw as food source. After each exposure time, the termite was rinsed in water to remove nematodes from their surface and the insect was transferred to a sterile Petri dish lined with moist paper. The number of nematodes inside each insect, alive or dead, was determined by dissection in Ringer's solution under a stereo-microscope. Ten *M. bellicosus* workers were tested for each combination of nematode isolate and exposure time. Each experiment was repeated four times.

4.2.4.2 Effect of nematode concentrations on termite mortality

Workers of *M. bellicosus* were exposed individually to EPN in 2 ml-Eppendorf tubes holding a small hole in the lid (approx. 0.6 mm diameter) to allow air exchange. Each tube was filled with 3 g moist sterilized sand priviously sifted with a 2-mm mesh sieve and adjusted to 10% moisture (w/w). 60 µl of distilled water containing the assigned nematode concentration (1, 5, 20, 40 or 100 IJ) was transferred into the tubes along with one worker of *M. bellicosus*. The control was only treated with 60 µl water. Termites were provided with a thin dried straw as food source. Upon insect transfer the tubes were sealed and kept in the dark at 25°C. Insect mortality was recorded after 48 h of exposure. Dead termites were removed from the tubes, rinsed with sterile water to eliminate nematodes from insect surfaces, and transferred to sterilized Petri dishes (padded with moist paper). Insect cadavers were dissected in Ringer's solution under a stereo-microscope to confirm parasitism. Per combination concentration x isolate three replicates of 10 insects were examined. The experiment was repeated twice in the same conditions.

4.2.4.3 Effect of exposure time on nematode infectivity against M. bellicosus

Workers of *M. bellicosus* were exposed individually to 100 IJ of each isolate suspended in 60 µl of sterile water pipetted into 2 ml-Eppendorf tubes filled with 3 g moist (10% w/w) sterilized sand (passed through a 2-mm mesh sieve) as described in the previous experiment. Insect mortality was assessed every two hours beginning 12 h after treatment until 90% mortality was achieved. Control was performed by applying only 60 µl of sterile water. Ten workers, each in a separate tube, were used per isolate. The experiment was repeated four times.

4.2.4.4 Reproduction potential in M. bellicosus

Dead insects from the previous experiment (4.2.4.3) were used to examine the multiplication potential of the selected isolates. Upon insect death 48 h after inoculation, ten insect cadavers were randomly selected, dissected in Ringer under stereo-microscope to confirm parasitism and the number of nematodes in the cadavers was counted to assess IJ penetration rate. Likewise, another ten insect cadavers were randomly selected and transferred individually onto White traps for reproduction potential in termite evaluation. The traps were kept at 25°C and checked daily. All IJ that emerged from a single cadaver over a period of 7 days after the first appearance of nematodes in the water were harvested and the total nematode number was evaluated. Thirty insects were used per isolate to perform the experiment and ten insects were randomly selected for penetration as well as reproduction evaluations. The experiment was performed twice in the same conditions with different batches of nematodes.

4.2.5 Data analysis

Bioassays that showed control mortality were discarded and further repeated. Only bioassays that showed no mortality were considered. In all cases, data from repeated experiments were similar and were therefore pooled for analysis. To stabilize the variance of means, data obtained as percentages were transformed (arcsine of the square root) prior to analysis of variance (ANOVA); non-transformed means are presented in figures. Data were subjected to ANOVA using SAS (SAS, 2001) and the differences between means were compared using Student-Newman-Keuls (SNK) test at $P < 0.05$. Linear regression analyses were performed using the REG procedure of SAS. Virulence assay data were used to calculate the Invasion

Time (IT_{50}), Lethal Concentration (LC_{50}) and Lethal Time (LT_{50}) values. These data were obtained using probit analysis in SPSS 16.0 (2007) for Windows. Differences among isolates were considered to be significant when the 95% confidence limits of IT_{50}, LC_{50} or LT_{50} values failed to overlap. Correlation analysis was also performed to determine the relationship between the nematode penetration and nematode production per insect in reproduction potential assay.

4.3 RESULTS

4.3.1 Host finding ability

Distances between termite and point of IJ inoculation had a negative impact on the efficacy of EPN isolates/species as indicated by negative slope of regression equations (Table 4.1). The relationship between nematode isolates/species and termite mortality varies according to distance between termite and point of IJ inoculation. Nematode host finding ability was therefore assessed separately for each tested distance (Table 4.2). Accordingly insect mortality differed significantly among EPN isolates/species at each tested distance except the distance of 5 cm (Table 4.2). The *H. sonorensis* isolates Aglali, Djidja1 and Yokon were able to induce 100% termite mortality at any tested distance whereas *H. sonorensis* isolates Ze1 recorded the lowest termite mortality at 15 and 20 cm with 33.3 and 26.7% termite mortality, respectively. Termite mortality differed significantly between the *H. indica* isolates LN2 and Ayogbe1 at 15 cm and 20 cm and the highest insect mortalities were recorded for isolate Ayogbe1 with 100 and 86.67% termite mortality, respectively (Table 4.2).

Table 4.1 Regression coefficient of an equation of $Y = \alpha + \beta X$, where Y is termite mortality (%) or IJ penetration rate (%), X is distance between insect and point of IJ inoculation, for thirty Beninean entomopathogenic nematodes applied at 1000 IJ to *Macrotermes bellicosus* at different depths (5, 10, 15 and 20 cm) in sand column assay.

Parameter	α^a	β^b	R^c	P^d
Termite mortality (%)	107.688 ± 3.186	-1.705 ± 2.33	0.559	<0.0001
IJ penetration rate (%)	2.107 ± 0.197	-0.097 ± 0.014	0.527	<0.0001

[a] Constant (mean ± SEM), [b] Slope (mean ± SEM), [c] Correlation, [d] Probability

Table 4.2 Mortality (% ± SEM) of *Macrotermes bellicosus* exposed to different *Heterorhabditis* species or isolates at 5, 10, 15 and 20 cm distance between insect and point of inoculation of the nematodes in sand column assay.

EPN species	Isolate	Mortality (% ± SEM)[a] at				Place of isolation
		5cm	10cm	15cm	20cm	
H. sonorensis	Kpedekpo	80±11.6 a	66.7±6.7 b	53.3±6.7 bc	53.3±17.6 bcd	Kpedekpo, Benin
	Ouere1	100 a	93.3±6.7 ab	93.3±6.7 ab	80 abcd	Adja-Ouere, Benin
	Ouere2	93.3±6.7 a	73.3±6.7 ab	66.7±6.7 abc	60 abcd	Adja-Ouere, Benin
	Hessa1	93.3±6.7 a	93.3±6.7 ab	93.3±6.7 ab	80±11.6 abcd	Hessa, Benin
	Hessa2*	100 a	100 a	100 a	93.3±6.7 ab	Hessa, Benin
	Aliho	93.3±6.7 a	93.3±6.7 ab	80±11.6 ab	80±20 abcd	Azohoue Aliho, Benin
	Kpanroun	93.3±6.7 a	93.3±6.7 ab	86.7±6.7 ab	60±11.6 abcd	Kpanrou, Benin
	Zagnanado	93.3±6.7 a	66.7±6.7 b	60±11.6 bc	40 cd	Zagnanado, Benin
	Akohoun	93.3±6.7 a	73.3±17.7 ab	60±11.6 bc	60±11.6 abcd	Akohoun, Benin
	Setto1	100 a	100 a	93.3±6.7 ab	66.7±6.7 abcd	Setto, Benin
	Setto2	93.7±13.3 a	86.7±13.3 ab	80 ab	66.7±13.3 abcd	Setto, Benin
	Setto3	93.3±6.7 a	86.7±6.7 ab	66.7±13.3 abc	60±13.3 abcd	Setto, Benin
	Ze1	100 a	66.7±6.7 b	33.3±6.7 c	26.7±6.7 c	Ze, Benin
	Ze3*	100 a	100 a	100 a	93.3±6.7 ab	Ze, Benin
	Ze4	93.3±6.7 a	93.3±6.7 ab	86.7±13.3 ab	66.7±6.7 abcd	Ze, Benin
	Akare	100 a	100 a	86±13.3 ab	80 abcd	Oko-Akare, Benin
	Zoundomey*	100 a	100 a	100 a	86.7±6.7 abc	Zoundomey, Benin
	Kissamey	100 a	100 a	66.7±6.7 abc	73.3±6.7 abcd	Kissamey, Benin
	Azohoue1	100 a	100 a	100 a	73.3±6.7 abcd	Azohoue Azongo, Benin
	Azohoue2*	100 a	93.3±6.7 ab	93.3±6.7 ab	93.3±6.7 ab	Azohoue Azongo, Benin
	Kemondji*	100 a	100 a	93.3±6.7 ab	86.7±13.3 abc	Kemondji, Benin
	Djidja1*	100 a	100 a	100 a	100 a	Djidja, Benin
	Djidja2	100 a	100 a	86.7±6.7 ab	40±11.6 cd	Djidja, Benin
	Yokon*	100 a	100 a	100 a	100 a	Yokon, Benin
	Aglali*	100 a	100 a	100 a	100 a	Aglali, Benin
	Kassehlo	100 a	100 a	93.3±6.7 ab	73.3±6.7 abcd	Kassehlo, Benin
	Dan	100 a	100 a	73.3±6.7 abc	46±6.7 bcd	Dan, Benin
	Avokanzoun	100 a	100 a	73.3±6.7 abc	53.3±13.3 bcd	Avokanzoun, Benin
H. indica	LN2	100 a	100 a	86.7±6.7 ab	80±11.5 abcd	Coimbatore, India
	Ayogbe1*	100 a	100 a	100 a	86.67±6.7 abc	Ayogbe, Benin
F value		1.04	4.35	5.14	4.80	
Probability		0.4386	<0.0001	<0.0001	<0.0001	

[a] Means (% ± SEM) within a column followed by the same letter are not significantly different (SNK's test; $P < 0.05$). SEM = Standard Error of Mean.

* Isolates selected for virulence studies.

4.3.2 Virulence

The virulence of selected isolates was evaluated on the basis of their ability to kill the insect as well as their potential to produce offspring inside workers of *M. bellicosus*.

4.3.2.1 Invasion time

Significant differences in IT_{50} were observed among selected isolates (non-overlap of the 95% confidence limits of the IT_{50}) (Table 4.3). The highest IT_{50} were recorded with *H. sonorensis* Djidja1, *H. sonorensis* Aglali and *H. indica* Ayogbe1 with IT_{50} = 5.96, 5.69 and 5.77 h, respectively, and these IT_{50} values were not significantly different. Likewise, the IT_{50} values were not significantly different for the *H. sonorensis* isolates Kemondji (5.05 h), Zoundomey (4.79 h), Hessa2 (4.30 h) and Yokon (4.02 h). Isolates *H. sonorensis* Ze3 (3.35 h) and Azohoue2 (3.67 h) did not differ as well. These latter isolates recorded the lowest values of IT_{50} (Table 4.3).

Table 4.3 Comparison of time-parasitism response of *Heterorhabditis* isolates from southern Benin against a subterranean termite, *Macrotermes bellicosus.*

Species	Isolate	Probit equation[a]	Chi² [b]	IT_{50}[c] (h)	95%CL[d]
H. sonorensis	Djidja1	Y=-2.46 + 0.41X	2.268	5.96 a	4.8 - 7.07
	Aglali	Y=-2.87 + 0.50X	1.377	5.69 a	4.65 - 6.71
	Yokon	Y=-1.45 + 0.36X	0.960	4.02 ab	2.21 - 5.22
	Hessa2	Y=-1.85 + 0.43X	0.709	4.30 ab	2.89 - 5.38
	Zoundomey	Y=-1.91 + 0.40X	4.020	4.79 ab	3.37 - 5.89
	Kemondji	Y=-2.03 + 0.40X	0.660	5.05 ab	3.75 - 6.16
	Azohoue2	Y=-2.03 + 0.55X	0.677	3.67 b	2.39 - 4.64
	Ze3	Y=-1.89 + 0.56X	0.384	3.35 b	1.96 - 4.32
H. indica	Ayogbe1	Y=-2.95 + 0.51X	1.155	5.77 a	4.72 - 6.77

[a] General responses of percentage parasitized insect (Y) as a function of exposure time (T).
[b] Chi², calculated values of chi-square;
[c] Time (expressed in hours) required to parasitize 50% of treated insects. IT_{50} values within a column followed by the same letter are not significantly different; based on non-overlapping 95% CL.
[d] 95% confident limits (CL) for the IT_{50}.

The number of IJ found inside parasitized hosts after each time of exposition was quite different among nematodes (Figure 4.1). The highest penetration rate per termite was registered for *H. sonorensis* isolate Ze3 followed by *H. sonorensis* isolate Azohoue2 with 11.43 and 9.98% after 12 h exposure time, respectively. *H. indica* Ayogbe1 and *H. sonorensis* Djidja recorded the lowest IJ penetration rate with 1.76 and 1.09% after 12 h exposure time, respectively.

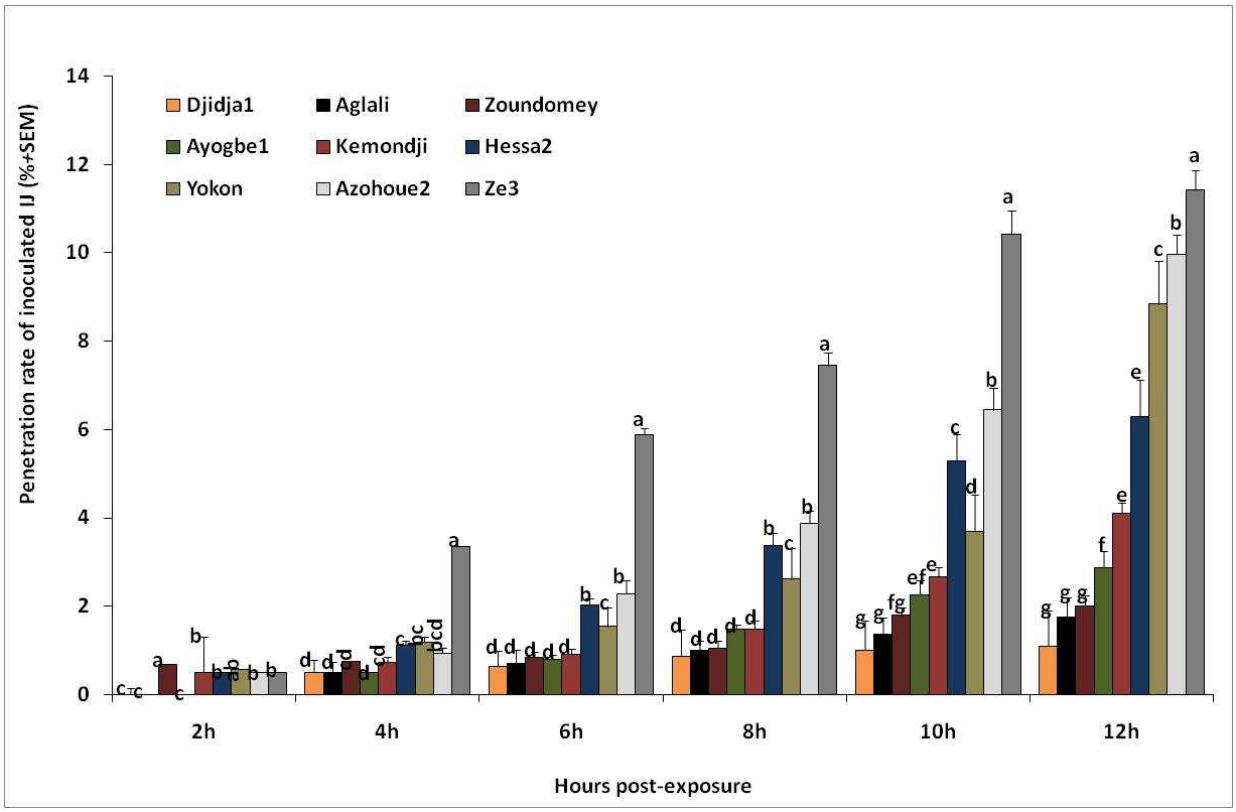

Figure 4.1 Penetration (% of inoculated IJ ± SEM) of infective juveniles (IJ) in *Macrotermes bellicosus* over exposure times (2, 4, 6, 8, 10 and 12 h) in invasion time assay of eight *H. sonorensis* isolates (Yokon, Hessa2, Aglali, Zoundomey, Azohoue2, Kemondji, Djidja and Ze3) and one *H. indica* isolate (Ayogbe1) from southern Benin. Vertical bars are standard error means. Bars headed by different letters denote significant differences among EPN isolates at the same hours post-exposure (SNK's test at $P < 0.05$).

4.3.2.2 Effect of nematode concentrations on mortality of Macrotermes bellicosus

Based on the non-overlap of the 95% confidence limits of the LC_{50}, no significant difference was observed in LC_{50} values among nematode isolates (Table 4.4). However insect mortality differed significantly among nematode isolates only at concentrations of 5 and 40 IJs (Figure 4.2). All tested nematode isolates induced 100% insect mortality for a concentration of 100 IJs. For all *H. sonorensis* isolates as well as for the local *H. indica* isolate (Ayogbe1), a concentration of 40 IJ/termite was enough to induce a termite mortality of at least 80% (Figure 4.2).

Table 4.4 Comparison of dose-mortality responses of *Heterorhabditis* nematode isolates from southern Benin against a subterranean termite, *Macrotermes bellicosus*.

Species	Isolate	Probit equation[a]	Chi² [b]	LC_{50} [c]	95%CL[d]
H. sonorensis	Yokon	Y=-0.88 + 0.05C	0.698	15 a	6.27 - 24.89
	Hessa2	Y=-0.64 + 0.06C	0.313	11 a	0.56 - 19.22
	Aglali	Y=-0.79 + 0.06C	0.843	14 a	4.34 - 23.06
	Zoundomey	Y=-1.3 + 0.1C	0.072	13 a	7.38 - 20.48
	Azohoue2	Y=-0.82 + 0.05C	1.141	16 a	6.17 - 28.11
	Kemondji	Y=-0.78 + 0.06C	2.046	14 a	4.15 - 23.22
	Djidja1	Y=-0.73 + 0.08C	1.056	10 a	1.8 - 17.29
	Ze3	Y=-0.86 + 0.1C	0.521	9 a	2.72 - 16.2
H. indica	Ayogbe1	Y=-0.65 + 0.06C	1.357	10 a	0.64 - 18.48

[a] General responses of percentage infected insect (Y) as a function of nematode concentration (C).
[b] Chi², calculated values of chi-square;
[c] Concentration (number of IJs per insect) required killing 50% of treated insects; LC_{50} values within a column followed by the same letter are not significantly different, based on non-overlapping 95% CL.
[d] 95% confident limits (CL) for the LC_{50}

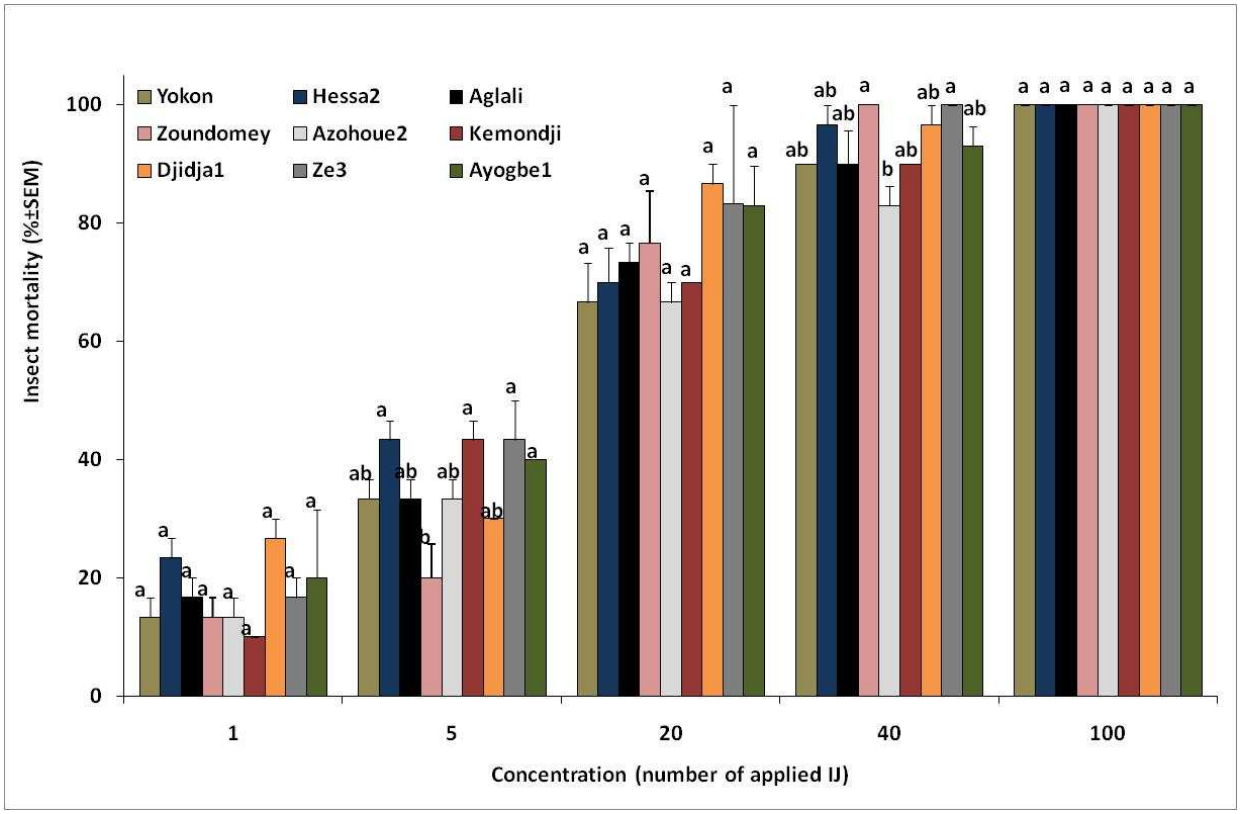

Figure 4.2 Mortality (% ± SEM) of *Macrotermes bellicosus* workers exposed to eight *H. sonorensis* isolates (Yokon, Hessa2, Aglali, Zoundomey, Azohoue2, Kemondji, Djidja and Ze3) and one *H. indica* isolate (Ayogbe1) from southern Benin at concentrations of 1, 5, 20, 40 and 100 IJ/termite. Vertical bars are standard error means. Bars headed by different letter denote significant differences among EPN isolates in termite mortality at the same concentration (SNK's test at $P < 0.05$).

4.3.2.3 Effect of exposure times on nematode infectivity against Macrotermes bellicosus

Based on the 95% confidence limits of the LT_{50} (Table 4.5), significant differences were observed among isolates. The shortest time was calculated for the *H. sonorensis* isolate Ze3 (23.30 h); the longest time was recorded for *H. sonorensis* Yokon (34.76 h). No difference was observed among the remaining isolates of *H. sonorensis* with LT_{50} values varying between 25.18 and 30 h. The LT_{50} value for the local *H. indica* isolate Ayogbe1 (24.07 h) was not significantly different from the value of these latter *H. sonorensis* isolates (Table 4.5).

Table 4.5 Comparison of time-mortality response of *Heterorhabditis* isolates from southern Benin against a subterranean termite, *Macrotermes bellicosus.*

Species	Isolate	Probit equation[a]	Chi²[b]	LT_{50}[c] (h)	95%CL[d]
H. sonorensis	Yokon	Y=-3.32 + 0.09T	1.332	34.76 a	28.97 - 41.36
	Hessa2	Y=-2.84 + 0.11T	2.992	25.18 ab	19.03 - 30.44
	Aglali	Y=-2.21 + 0.08T	3.098	28.29 ab	20.73 - 34.8
	Zoundomey	Y=-2.91 + 0.11T	2.497	27.03 ab	21 - 32.5
	Azohoue2	Y=-2.46 + 0.08T	2.461	29.43 ab	22.55 - 35.8
	Kemondji	Y=-3.25 + 0.11T	1.762	30 ab	24.34 - 35.67
	Djidja1	Y=-2.92 + 0.87T	2.560	25.55 ab	19.51 - 30.80
	Ze3	Y=-3.19 + 0.14T	2.584	23.30 b	17.57 - 28.12
H. indica	Ayogbe1	Y=-2.65 + 0.11T	4.586	24.07 ab	17.56 - 29.33

[a] General responses of percentage insect mortality (Y) as a function of exposure time (T).
[b] Chi², calculated values of chi-square;
[c] Time (expressed in hours) required killing 50% of treated insects; LT_{50} values within a column followed by the same letter are not significantly different, based on non-overlapping 95% CL.
[d] 95% confident limits (CL) for the LT_{50}.

4.3.2.4 Reproduction potential in Macrotermes bellicosus

The potential for multiplication of the EPN in *M. bellicosus* workers differed significantly among isolates (F = 3.72; df = 8; P < 0.0013). The highest multiplication was observed for *H. sonorensis* Yokon (20,213 IJ/insect) followed by *H. sonorensis* Ze3 (19,368 IJ/insect) (Figure 4.3). This multiplication was significantly different from the multiplication registered for *H. sonorensis* Azouhoue2 (10,511 IJ/termite) and *H. sonorensis* Kemondji (9,766 IJ/termite).

The correlation between the penetration rates of inoculated IJs and the number of emerged offspring per insect was not significant (r = 0.3196; P > 0.402). The only one isolate located on the regression line (not shown) was *H. indica* isolate (Ayogbe1) for which the general response of number of emerged IJ per insect (Y) as a function of number of penetrated IJ (X) were expressed as Y= 12027 + 238.70*X.

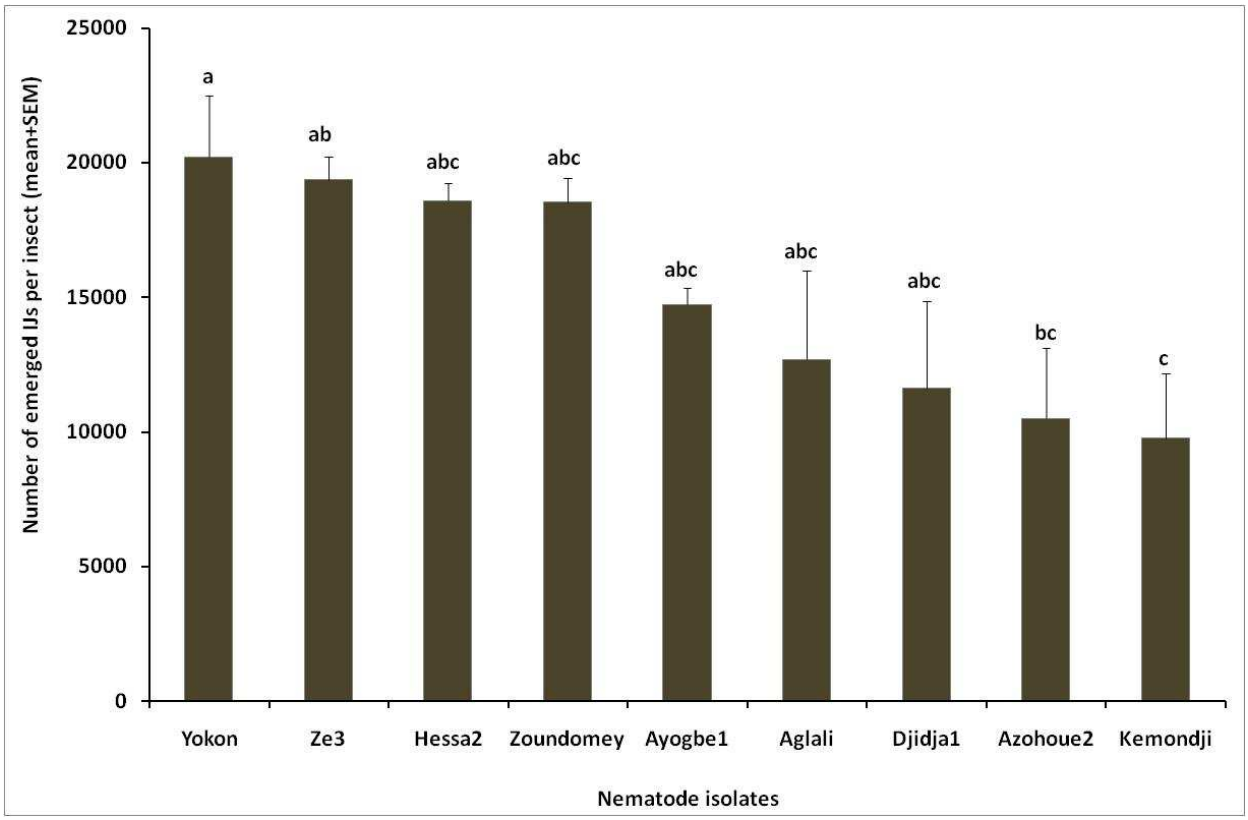

Figure 4.3 Mean multiplication (mean ± SE) in *Macrotermes bellicosus* workers of eight isolates of *Heterorhabditis sonorensis* (Yokon, Hessa2, Aglali, Zoundomey, Azohoue2, Kemondji, Djidja and Ze3) and one isolate of *H. indica* (Ayogbe1) from southern Benin. Vertical bars are standard error means. Bars headed by different letter denote significant differences among EPN isolates (SNK's test at $P < 0.05$).

4.4 DISCUSSION

Quite often, programmes aiming at the development of strategies for the biological control of insects start with laboratory bioassays. These assays screen local EPN for various beneficial traits in order to identify potential biocontrol candidates for pest control and to reduce the number of strains or species that need to be tested in the field (Mannion & Jansson, 1992; Patterson Stark & Lacey, 1999; Shapiro & McCoy, 2000). The results of my bioassays indicated that isolates of both *H. sonorensis* and *H. indica* exhibit a cruiser type of insect search strategy. Isolates of both species were capable, to various extents, of migrating, infecting and killing *M. bellicosus* workers in sand columns of 20cm in a period of three days. Campbell and Lewis (2002) reported a widely foraging strategy (cruiser) for *H. indica*. Our data corroborate the finding of El-Assal *et al.* (1997) that all the EPN isolates the authors tested, could kill *G. mellonella* larvae up to 10 cm within 48 h and up to 15 cm within 72 h.

Even though it is predictable that increased distances between insect and point of IJ inoculation affected the efficacy of EPN isolates, we obtained satisfactory results with the *H. sonorensis* isolates Yokon, Aglali and Djidja1, which all caused 100% mortality to *M. bellicosus* workers within 72 h when inoculated at a distance of 20 cm in a sand column. LN2, the exotic isolate of *H. indica*, did not show a high host finding ability compared to the indigenous isolate of the same species (Ayogbe1).

The results from our studies on the invasion time of *M. bellicosus* also showed significant differences among selected isolates. This is in agreement with Glazer (1992) who revealed a large variability in invasion capacities among different nematode isolates, suggesting that measuring invasion time could be a useful strategy for the selection of virulent strains of EPN. Hominick and Reid (1990) proposed the use of invasion efficiency as a direct measure of nematode infectivity. These authors assumed that the nematodes with the greatest efficacy against a target insect would have the highest invasion efficiency. In this respect, based on their lowest values of IT_{50} (3.35 h and 3.67 h, respectively) and their highest penetration rates (11.4% and 10%, respectively after 12 h exposure) isolates Ze3 and Azohoue2 of *H. sonorensis* could be the most virulent among the tested isolates.

All tested isolates of *H. sonorensis* and *H. indica* appeared to have non-differing LC_{50} values ranging from 9 to 16 IJs/termite. Interestingly, 40 IJs/termite were enough to cause 80% mortality to *M. bellicosus* workers. These data do not support those presented by Wang *et al.* (2002) who found that under laboratory conditions, continued exposure of termites to *H. indica* at 400 IJ/termite resulted after four days in 58 and 73% mortality of workers of *Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* (Shiraki), respectively. Contrary to the $LC_{50,}$ significant differences between isolates were observed with respect to the lethal time (LT_{50}) showing that *M. bellicosus* exhibits a time-dependent susceptibility to the tested nematode isolates. The lowest LT_{50} was estimated for *H. sonorensis* Ze3 (LT_{50}=23.30 h) that also showed the highest penetration rate. This suggests that insects parasitized with a higher number of nematodes take a shorter time to die. High infection rates increase toxins produced by developing nematodes (Burman, 1982) and their symbiotic bacteria (Akhurst & Boemaere, 1990) hence will enhance septicaemia and kill the insect host more rapidly. *H. sonorensis* Yokon exhibited the highest LT_{50} value (34.76 h) in spite of its high penetration rate into *M. bellicosus* compared to *H. sonorensis* Djidja1 for example. This suggests that, along with penetration, which is obviously crucial in the pathogenic process, there are also some other virulence factors involved in the deaths of the insects. When a nematode penetrates in the

insect, it may take various times before releasing the bacteria into the insect haemocoel (Ensign & Ciche, 2000). This may increase the time of insect death independently of nematode penetration rate into the insect. It has been reported by Divya and Sankar (2009) that *H. indica* needed 36 h to cause 50% mortality to the termite species *Odontotermes obesus.* However, the *H. indica* isolate (Ayogbe1) in our study needed only 24.07 h to induce 50% mortality to *M. bellicosus*.

Nematode reproductive capacity in the host insect is of major importance when aiming at recycling of the nematode after its application in the field. If nematode reproduction can occur in the target insect, then long-term management might be achievable. All selected isolates were able to reproduce in *M. bellicosus*. Correlation analysis showed that the reproduction potential of nematode isolates does not depend inevitably on the number of penetrated IJs inside the host. This suggests that beside the number of IJs that penetrate the host, other features like the amount of bacteria released per IJ and host size might be determining in nematode development and offspring production potential (Blinova & Ivanova, 1987; Flanders *et al*., 1996; Grewal, *et.al*., 1997; YU, 2009). The *H. sonorensis* isolate Yokon was the most reproductive in *M. bellicosus* with 20213 IJs/termites followed by *H. sonorensis* Ze3 with 19368 IJs/termite. The average yield of IJ (15,118/termite) of all tested isolates was considerably less than for other hosts, such as the corn earworm (311,000 IJ/insect) or *G. mellonella* (200,000 IJ/insect) (Cabanillas & Raulston, 1994; Dutky *et al*., 1964). In general, nematode yield is proportional to insect host size (Blinova & Ivanova, 1987; Flanders *et al*., 1996), but yield per milligram insect (within host species) and susceptibility to infection is usually inversely proportional to host size or age (Dutky *et al*., 1964; Shapiro *et al*., 1999).

In summary, the results show that all tested Beninese *Heterorhabditis* isolates are pathogenic to the citrus termite pest *M. bellicosus*; however, isolate Ze3 of *H. sonorensis* is the most virulent. However, there are many other biological factors that can affect the final choice of the tested EPN species/isolates for the control of *M. bellicosus.* Abiotic factors, such as UV radiation, fluctuating soil temperature and moisture content, and antagonists in the field could prevent the EPN from realizing their full potential as bio-insecticides (Kaya & Koppenhöfer, 1996; Smits, 1996; Gray, 1988; Grewal, 2011). Further studies in field-simulated conditions in the laboratory, in glasshouses and in the field are still needed before making a choice of the promising isolates for biological control of *M. bellicosus*.

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5

Comparative susceptibility of *Macrotermes bellicosus* and *Trinervitermes occidentalis* (Isoptera: Termitidae) to entomopathogenic nematodes from Benin

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ABSTRACT

The differential susceptibility of two termite species *Macrotermes bellicosus* and *Trinervitermes occidentalis* to four entomopathogenic nematodes (EPN) isolates from Benin, *Heterorhabditis indica* Ayogbe1, *H. sonorensis* Azohoue2, *H. sonorensis* Ze3 and *Steinernema* sp. Bembereke was bio-assayed in the laboratory. Soldiers of both *M. bellicosus* and *T. occidentalis* were similarly susceptible, but more susceptible than workers. Forty eight hours post-exposure of workers of *M. bellicosus* to 50 infective juveniles (IJ) of *H. indica* Ayogbe1, *H. sonorensis* Azohoue2, *H. sonorensis* Ze3 and *Steinernema* sp Bembereke per termite resulted in 96.3%, 87.9%, 94.5% and 75% mortality, respectively whereas under the same conditions, these EPN isolates caused 91.7%, 98.5%, 75% and 95% mortality of workers of *T. occidentalis*. Soldiers of *M. bellicosus* were the most invaded with 13.2% to 18.6% of applied IJ. Based on concentration-mortality data, the isolates *H. indica* Ayogbe1 and *H. sonorensis* Ze3 were more virulent to *M. bellicosus* with LC_{50} values of 11 IJ, whereas *Steinernema* sp. Bembereke was the most virulent to *T. occidentalis* with LC_{50} values of 12 IJ. However, none of these isolates showed the highest penetration rate. All tested EPN isolates can recycle in both *M. bellicosus and T. occidentalis.* Our EPN repellent-dispersing assay did not show evidence that *M. bellicosus* and *T. occidentalis* would be able to detect the presence of IJ of any EPN isolates/species. However, it was observed that nematode dispersal occurred by infected termites or phoresis.

5.1 INTRODUCTION

Termites are soil-inhabiting arthropods that cause serious losses to most tropical dry land crops, including fruits (Wood & Cowie, 1988). Since the major constituent of the diet of termites is cellulose, the insects tunnel through stems and weaken them by consuming the carbohydrate. Eventually the insects cause their collapse or give access to fungi and other disease causing agents (Femi-Ola *et al*., 2007; Kumar & Pardeshi, 2011).

In Benin, citrus (*Citrus sinensis*) is the second most produced and exported fruit crop after pineapple (FAO, 2001). Losses may fluctuate according to harvest times, and vary between 25% (March-July) and 50% (November-January) (UEMOA, 2008). The most frequent and damaging termite species in Southern Benin citrus orchards belong to the subfamily Macrotermitinae, *viz. Macrotermes bellicosus* (Smeathman), *Amitermes guineensis* (Sands), *Ancistrotermes crucifer* (Sjöstedt) and *Trinervitermes occidentalis* (Sjöstedt) (ESCiP-Benin, 2011). To reduce damage caused by these insects, Beninese citrus growers often rely on synthetic chemicals like fipronil, deltametrin, dimethoate and fenitrothion (H.K. Baimey, pers. com.). Although intensive pesticide use has increased crop production, the long-term and highly concentrated application of pesticides may contaminate the yield of crops and pose a serious danger to the agro-ecosystem and human health (Rola & Pingali, 1993). Due to increasing concerns about these side effects, alternative means for termite control are being investigated (Grace, 1997).

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae, along with their associated symbiotic bacteria of the genera *Xenorhabdus* and *Photorhabdus*, respectively, are promising alternatives to chemical control (Kaya & Koppenhöfer, 2004). The infective juveniles (IJ) of EPN penetrate via natural openings into the insect haemocoel where they release the symbiotic bacteria, thereby causing septicaemia and death of the host, usually within 48 h after infection (Kaya & Gaugler, 1993). The nematodes feed on the bacteria, reproduce in the insect and eventually IJ emerge from the host cadaver to infect new hosts. The efficiency of EPN in locating, infecting and killing an insect, is profoundly affected by the host (Gouge *et al.*, 1999).

When comparing both the host searching behaviour and the virulence of Beninese heterorhabditid isolates in bioassays (Chapter 4), I demonstrated that some of them were promising control agents against *M. bellicosus*. However, no information is available on the

effects of EPN on *T. occidentalis*, a termite species frequently found in the same field with *M. bellicosus*. I hypothesised that *T. occidentalis*, when exposed to EPN, may react in a way different from *M. bellicosus*. Therefore, the aim of this study was to gain greater understanding of the nematode - termite species interaction. I here report on the differential of susceptibility of two termite species *M. bellicosus* and *T. occidentalis* to EPN infection. Hereby, I compared virulence of three indigenous EPN species (*Heterorhabditis sonorensis*, *H. indica* and an undescribed *Steinernema* sp.) towards two castes (worker and soldier) of both termite species in relation to penetration efficiency of IJ and production rates of IJ in both termite species/castes. The abilities of nematodes to parasitize termites at different concentrations of IJ and to recycle were also investigated, as well as the repellent effect of nematodes on termites.

5.2 MATERIALS AND METHODS

5.2.1 Source and maintenance of nematodes and insects

Heterorhabditid isolates involved in this study (Table 5.1) were selected because of their ability of migrating, infecting and killing *M. bellicosus* in a previous experiments (Chapter 4), a population of an undescribed Beninese *Steinernema* sp. was included to broaden the scope of EPN. This latter species was collected at Bembereke village during an extra survey conducted in northern Benin in 2012. All isolates were multiplied in last instar larvae of *Galleria mellonella* (0.22 ± 0.018 g). Nematodes were harvested within the first week of emergence from modified White traps (Kaya & Stock, 1997) and used within 3 weeks of storage at 13-15°C. Before use, IJ were acclimated at room temperature (25°C) for two hours after which their viability (on the basis of movement) was checked under a stereomicroscope. Concentrations were calculated according the technique of Navon and Ascher (2000).

Workers and soldiers of *M. bellicosus* and *T. occidentalis* were collected from a citrus field near the University of Parakou in Benin. To collect workers and soldiers of *M. bellicosus*, the termite mounds were broken at their base, covered with dried straw, and left for 3-4 hours. Thereafter, termite-infested straws were taken to the laboratory where the termites were collected and transferred into 1000-ml plastic boxes containing small pieces of moist paper (food source) and moist sand collected from the termite nest. The termite species can easily be distinguished from each other; mounds made by *T. occidentalis* are much smaller than those

of *M. bellicosus*. *Trinervitermes occidentalis* were collected directly from the broken mounds and put in a 1000-ml plastic box, which was transferred to the laboratory. The boxes were maintained in the dark at 25°C and 75-80% RH for 24 h before termites were used in the experiments. Workers and soldiers of *M. bellicosus* weighed 38 ± 3 mg and 86 ± 6 mg, respectively; those of *T. occidentalis* weighed 10 ± 2 mg and 2 ± 0.2 mg, respectively.

Table 5.1 Beninese entomopathogenic nematode isolates used in the present study.

Species	Isolates	Accession numbers	Year of isolation	Original locality	Vegetation
H. indica	Ayogbe1	KF723816	2011	Ayogbe, Zou	Mango
H. sonorensis	Azohoue2	KF723809	2010	Azohoue, Atlantique	Lemon
H. sonorensis	Ze3	KF723828	2011	Ze, Atlantique	Palm tree
Steinernema sp.	Bembereke	-	2012	Bembereke, Borgou	Forest

5.2.2 Response of workers and soldiers of *Macrotermes bellicosus* and *Trinervitermes occidentalis* to entomopathogenic nematodes

Tissue culture plates with 24 wells (3.5 ml/well) were filled with 1 ml dried and sterilized (80°C, 72 h) sand (< 2 mm). Fifty IJ suspended in 80 µl water were pipetted on top of the sand. Wells of the control plate received only 80 µl distilled water. In preliminary observations, a volume of 80 µl was found to provide sufficient moisture for optimal nematode virulence. Each of the 24 wells received either one worker or one soldier of either *M. bellicosus* or *T. occidentalis*. Plates, covered with a lid, were placed in a plastic bag to maintain moisture and were incubated in the dark at 25°C. A split-split-plot design with EPN as main factor, termite species as sub-factor, and caste as sub-sub-factor was used, with three replicates (plates) per treatment (EPN isolate x Termite species x Caste). To compare the response to EPN infection of workers and soldiers of both termite species, insect mortality was recorded 60 h post inoculation; virulence rates were calculated per plate (replicate). The experiment was conducted twice with different batches of nematodes. Penetration efficiency was estimated by selecting randomly 15 cadavers of either workers or soldiers of both termite species among dead insects. These cadavers were individually washed with distilled water to remove nematodes on their body. One day later they were individually dissected in Ringer's solution. Nematodes observed in each cadaver were counted. The experiment was performed

twice in the same conditions. To assess the effect of termite species/castes on the reproduction potential of the nematodes, the nematode offspring per insect was evaluated based on procedures described by Shapiro-Ilan (2001). In brief, 15 insect cadavers were randomly selected (see penetration efficiency). Dead cadavers were individually washed and transferred onto water traps (Kaya & Stock, 1997), which were kept in the dark at 25°C. All IJ that emerged from an individual cadaver over a period of 7 days after the first appearance of nematodes were counted and the number of nematodes per milligram of insect was calculated. The experiment was performed twice in the same conditions with different batches of nematodes.

5.2.3 Effect of EPN-concentration on susceptibility of *Macrotermes bellicosus* and *Trinervitermes occidentalis*

In the experiments described above, soldiers of both termite species were similarly susceptible to EPN, but more susceptible than workers. Therefore, only workers were used in an experiment in which the differential susceptibility of *M. bellicosus* and *T. occidentalis* to EPN isolates/species was examined over a range of nematode concentrations. Each well of 24-well plates was filled with 1 ml dried and sterilized (80°C, 72 h) sand (< 2 mm). One worker of *M. bellicosus* or *T. occidentalis* was added to each well where they were exposed to 5, 10, 25, 50 or 100 IJ suspended in 80 µl distilled water. The control plates received only 80 µl distilled water. Plates, covered with their lids, were placed in plastic bags to maintain moisture; they were incubated in the dark at 25°C. Three plates (replicates) were used per treatment (EPN x Concentration x Termite). They were arranged in a completely randomized design. The experiment was performed twice under the same conditions. Insect mortality was recorded 60 h post-inoculation. Dead insects were examined for nematode presence by dissection.

5.2.4 Nematode repellent effect and termite ability to disperse nematodes

Two identical plastic boxes A and B (6 and 8 cm in diameter at the base and top, respectively; 4 cm deep) were connected with transparent tubing (15 cm long, 1 cm inner diameter) near the base. Both boxes were filled with 40 cm^3 sterilized (80°C, 72 h) sand (< 2mm) adjusted to 10% moisture content (w/w), yielding a soil surface of 38.5 cm^2. The nematodes (Table 5.1) were pipetted on top of the soil in boxes B at a dose of 38,500 IJ suspended in 1 ml water (i.e. a density of 1000 IJ/cm^2 or 962.5 IJ/cm^3). Controls received only 1 ml water in box B. Boxes

A received 100 workers along with five soldiers of *M. bellicosus* or *T. occidentalis* either immediately after nematode inoculation in box B or one week later. Pieces of straw (2 g) were added as food source for termites in boxes A. The boxes were covered with their lids, placed in a plastic bag to maintain moisture, and incubated in the dark at 25°C.

Treatments (Termite species x Nematode isolate x Termite application date) were arranged in a completely randomised design with six replicates. The experiment was performed twice with all aforementioned EPN isolates. To assess the nematode repellent effect, the location of the termites was checked every two hours during the first eight hours, and daily up to 4 or 7 days for *M. bellicosus* and *T. occidentalis*, respectively. This difference in time was chosen because dead termites were found in the *M. bellicosus* control 4 days after inoculation. The repellent effect was evidenced when no termites entered the B boxes.

To assess the ability of termites to disperse nematodes, we hypothesized that if termites that reached box B would return back to box A, they might be infected by EPN or carry externally nematodes (phoresis) which could be deposited in box A. Three A boxes of each combination nematode-termite were baited 4 or 7 days after application of *M. bellicosus* or *T. occidentalis* with 5 *G. mellonella* larvae. The remaining three A boxes were kept for an additional 7 days before being baited by the same number of *G. mellonella* larvae. In all cases, the boxes were kept in the dark at room temperature (25°C). Larvae mortality was recorded 72 h later. Dead larvae were dissected to ascertain the presence of EPN.

5.2.5 Statistical analyses

Average termite mortality was calculated and corrected by comparing proportion of dead insect between treatments and control (< 10%) (Abbott, 1925). Data from repeated bioassays were similar and were therefore pooled for analysis. Data expressed in percentage were normalized using arcsine transformation. Data on virulence and reproductive capability were subjected to multi-factor analysis of variance (ANOVA) using PROC GLM (SAS Institute, version 8). Differences between means were compared using Student Newman Keuls (SNK) multiple means comparison procedure at $P < 0.05$. To determine the influence of termite species on nematode penetration efficiency, susceptibility of castes to nematode penetration was compared between termite species using Mann-Whitney U-test; Kruskal-Wallis analysis was performed for comparing the four nematodes isolates, as data could not be normalized.

Differences between isolates were then determined by comparing isolates per pairs (Mann-Whitney U-test). Data from concentration-mortality assays were analysed using probit analysis in SPSS 16.0 (2007) for Windows. Lethal concentration values (LC_{50}) were determined. Differences among EPN isolates/species were considered to be significant when the 95% fiducial limits of LC_{50} values failed to overlap.

5.3 RESULTS

5.3.1 Response of workers and soldiers of *Macrotermes bellicosus* and *Trinervitermes occidentalis* to entomopathogenic nematodes

A significant difference in termite mortality was recorded between termite castes, but not between EPN isolates and termite species (Table 5.2). The interaction between EPN isolates and termite species as well as the interaction between EPN isolates and caste of termites were not significant. Effects of EPN isolates on termite species/caste mortality were therefore analysed separately.

Table 5.2 Single and interaction effects of Beninese isolates of entomopathogenic nematode species (EPN) *Heterorhabditis indica* Ayogbe1, *H. sonorensis* Azohoue2, *H. sonorensis* Ze3 and *Steinernema* sp. Bembereke applied at a concentration of 50 IJ/insect on the mortality of workers and soldiers of two termite species *Macrotermes bellicosus* and *Trinervitermes occidentalis*. Insect mortality was recorded 60 h post infection at 25°C.

Source of variations	df	F	P
EPN-isolates	3	0.2	0.8967
Castes	1	85.9	<0.0001***
Termite species	1	0.01	0.9366
EPN-isolates x Castes	3	0.04	0.9887
EPN-isolates x Termite species	3	0.51	0.6749
Error	80	-	-

***=significant at P<0.001

Significant differences in termite mortality were observed between EPN isolates inoculated to workers of *M. bellicosus* ($F = 11.32$; df = 3, 20; $P < 0.0001$) or *T. occidentalis* ($F = 16.38$; df = 3, 20; $P < 0.0001$) (Figure 5.1). The isolates Ayogbe1 (96.3%) and Ze3 (94.9%) caused the

highest mortality to workers of *M. bellicosus*, whereas the highest mortalities of workers of *T. occidentalis* were recorded for Azohoue2 (98.5%) and Bembereke (95%). Unlike workers of both termite species, mortality recorded for soldiers did not differ significantly among EPN isolates, neither for *M. bellicosus* (F = 0.15; df = 3, 20; *P* = 0.9293) nor for *T. occidentalis* (F = 0.12; df = 3, 20; *P* = 0.9469) (Figure 5.1). Soldier mortalities ranged from 86.1 to 100% for *M. bellicosus* and 79.4 to 100% for *T. occidentalis*.

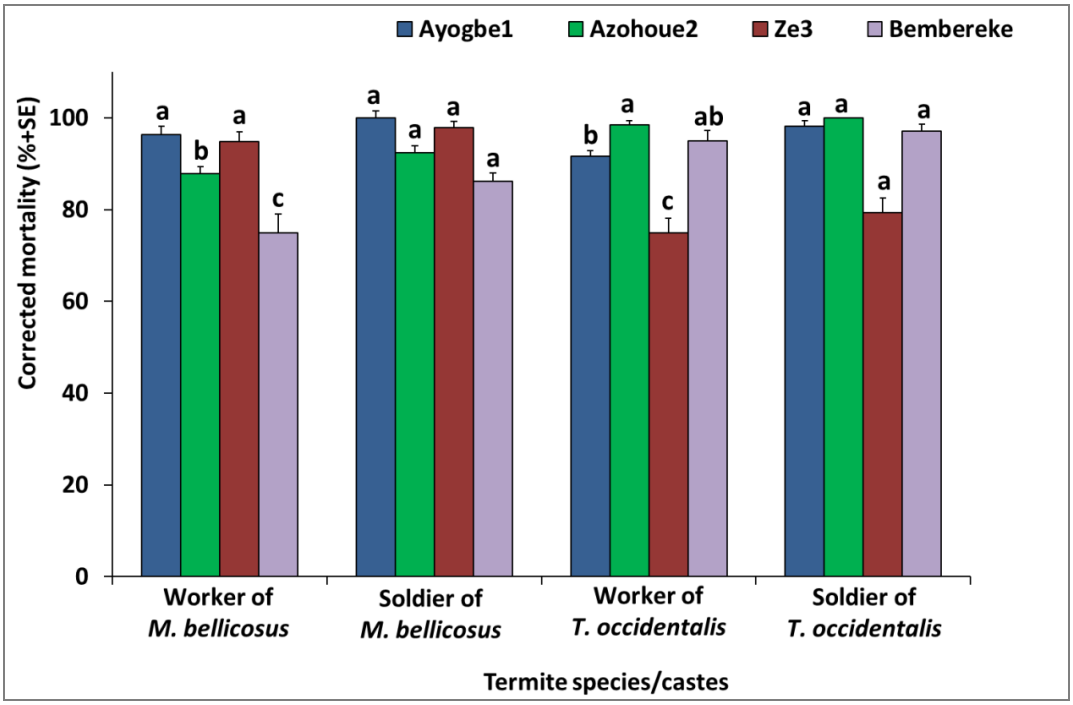

Figure 5.1 Mean corrected mortality (% ± SEM) of workers and soldiers of *M. bellicosus* and *T. occidentalis* caused by *Heterorhabditis indica* Ayogbe1, *H. sonorensis* Azohoue2, *H. sonorensis* Ze3 and *Steinernema* sp. Bembereke applied at a concentration of 50 IJ/insect. Mortality was recorded at 25°C after 48 h post infection. Different letters on the bar indicate significant differences among nematode isolates at P<0.05 (SNK's test).

Mann-Whitney U-analyses revealed significant differences in number of penetrating IJ between castes of *M. bellicosus* and *T. occidentalis* (Table 5.3). The highest penetration rates were always observed with soldiers of *M. bellicosus (*13.2-18.6%) followed by workers of the same termite species (5.2-8.8%) (Table 5.3). The Kruskal-Wallis analysis also showed significant differences among nematode isolates in number of IJ invading workers of *M. bellicosus* (df = 3, χ^2 = 9.553; *P* = 0.023), soldiers of *M. bellicosus* (df = 3, χ^2 = 9.213; *P* = 0.027) and soldiers of *T. occidentalis* (df = 3, χ^2 = 10.567; *P* = 0.014); no significant difference was observed among IJ penetrating workers of *T. occidentalis* (df = 3, χ^2 = 7.281;

P = 0.063) (data not shown). Consequently, for both *M. bellicosus* and *T. occidentalis*, the highest penetration rates of IJ were observed with the isolate Azohoue2 in worker (8.8 and 18.6%) and soldier castes (1-2%), respectively.

Table 5.3 Pairwise comparisons (Mann-Whitney U-test) of penetration efficiency (%) in *Macrotermes bellicosus* and *Trinervitermes occidentalis* of *Heterorhabditis indica* Ayogbe1, *H. sonorensis* Azohoue2, *H. sonorensis* Ze3 and *Steinernema* sp. Bembereke applied at 50 IJs/insect. Different capitals within lines denote significant differences among caste; different small letters within columns indicate significant differences among nematode isolates for a given caste (P<0.05, SNK's test).

Caste of *M. bellicosus* Vs Caste of *T. occidentalis*	EPN-isolates	Penetration into (%±SE)		U	Differences	
		M. bellicosus	*T. occidentalis*		Z	P
worker vs worker	Ayogbe1	5.2±0.4 Ab	1.4±0.1 Ba	0	-5.545	0.00***
	Azohoue2	8.8±1 Aa	2±0.2 Ba	5	-5.311	0.00***
	Ze3	6.4±0.7 Aab	1.5±0.1 Ba	4	-5.373	0.00***
	Bembereke	5.9±0.3 Ab	1.4±0.1 Ba	0	-5.524	0.00***
soldier vs soldier	Ayogbe1	13.2±0.8 Ab	1±0.1 Bb	0	-5.524	0.00***
	Azohoue2	18.6±1.6 Aa	1.4±0.1 Ba	0	-5.493	0.00***
	Ze3	13.9±0.9 Ab	1.1±0.1 Bab	0	-5.556	0.00***
	Bembereke	14.5±0.4 Aab	1.1±0.1 Bab	0	-5.524	0.00***
worker vs soldier	Ayogbe1	5.2±0.4 Ab	1±0.1 Bb	0	-5.579	0.00***
	Azohoue2	8.8±1 Aa	1.4±0.1 Ba	0	-5.500	0.00***
	Ze3	6.4±0.7 Aab	1.1±0.1 Bab	0	-5.562	0.00***
	Bembereke	5.9±0.3 Ab	1.1±0.1 Bab	0	-5.551	0.00***
soldier vs worker	Ayogbe1	13.2±0.8 Ab	1.4±0.1 Ba	0	-5.491	0.00***
	Azohoue2	18.6±1.6 Aa	2±0.2 Ba	0	-5.432	0.00***
	Ze3	13.9±0.9 Ab	1.5±0.1 Ba	0	-5.474	0.00***
	Bembereke	14.5±0.4 Aab	1.4±0.1 Ba	0	-5.498	0.000***

***=significant at P<0.001

Mann–Whitney U-test at P< 0.05; SE: standard error.

Multifactor analysis of variance revealed that the number of IJ produced per milligram insect (IJ/mgi) differed significantly among EPN isolates, castes and termite species (Table 5.4). The two-way interactions EPN-isolates x Castes and EPN-isolates x Termite species were also significant. The effect of termite species or castes on reproductive capability of EPN was assessed separately. Significant differences were observed among EPN isolates in number of IJ produced per milligram insect by workers (F = 15.53; df = 3, 36; *P* < 0.0001) and soldiers (F = 10.56; df = 3, 36; *P* < 0.0001) of *M. bellicosus*, as well as by workers (F = 9.76; df = 3, 36; *P* < 0.0001) and soldiers (F = 108.35; df = 3, 36; *P* < 0.0001) of *T. occidentalis* (Figure

5.2). The isolate Ze3 was the most productive among nematode isolates with 1370.8 and 566.5 IJ/mgi in soldiers and workers of *M. bellicosus*, respectively, 45.4 and 74.6 IJ/mgi in soldiers and workers of *T. occidentalis*, respectively.

Table 5.4 Single and interaction effects on number of infective juveniles (IJ) per milligram insect of Beninese entomopathogenic nematode (EPN) isolates *Heterorhabditis indica* Ayogbe1, *H. sonorensis* Azohoue2, *H. sonorensis* Ze3 and *Steinernema* sp. Bembereke produced by workers and soldiers of two termites species *Macrotermes bellicosus* and *Trinervitermes occidentalis*. Nematodes were applied at a concentration of 50 IJ/insect and mortality was recorded 60 h post infection at 25°C.

Source of variations	df	F	P
EPN-isolates	3	22.52	<0.0001***
Castes	1	357.41	<0.0001***
Termite species	1	1726.76	<0.0001***
EPN-isolates x Castes	3	3.69	0.0135*
EPN-isolates x Termite species	3	14.28	<0.0001***
Error	144	-	-

*=significant at P<0.05; ***=significant at P<0.001

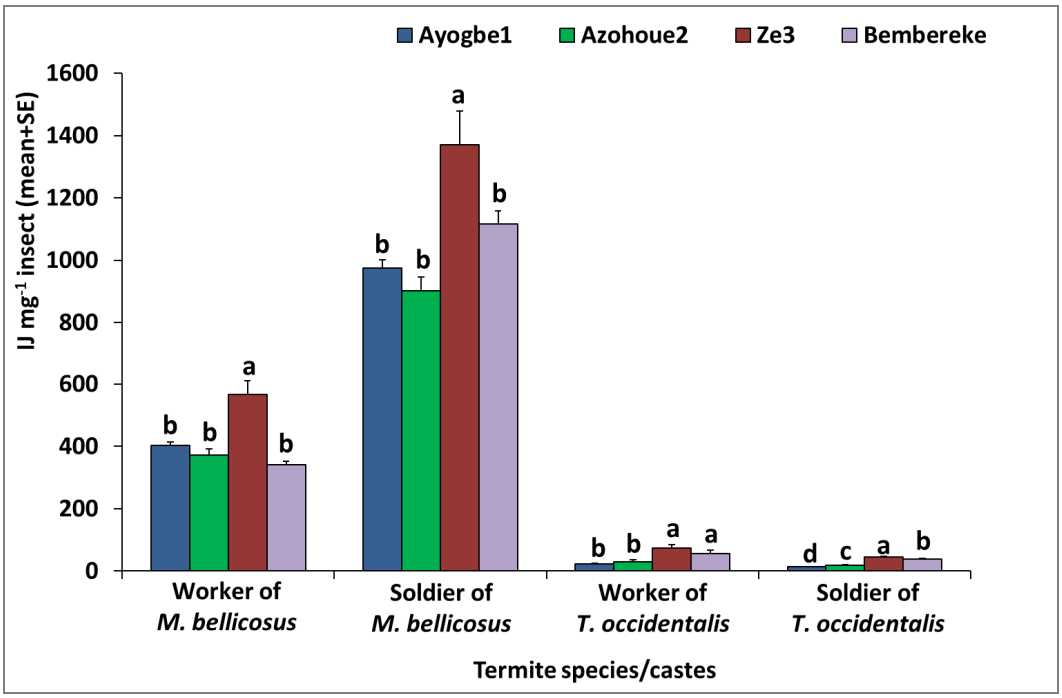

Figure 5.2 Yield per milligram insect (mean ± SE) of infective juveniles (IJ) of four entomopathogenic nematode isolates (Ayobe1, Azohoue2, Ze3 and Bembereke) produced in two termite species/castes (workers and soldiers of *M. bellicosus* and *T. occidentalis*). Nematodes were applied at a concentration of 50 IJ/insect. Different letters indicate significant differences among yields of the four nematode isolates at P < 0.05 (SNK's test).

5.3.2 Effect of EPN-concentration on susceptibility of *Macrotermes bellicosus* and *Trinervitermes occidentalis*

Mortality of termites differed significantly among EPN isolates (F = 7.79; df = 3, 200; $P < 0.0001$), termite species (F = 5.43; df = 1, 200; $P = 0.0207$), and nematode-concentrations (F = 1052.58; df = 4, 200; $P < 0.0001$). The three ways interaction EPN-isolates x Termite species x Nematode-concentration was also significant (F = 5.11; df = 12, 200; $P < 0.0001$) (data not shown). Three ways interaction results indicated *M. bellicosus* (62.8 %) was more susceptible than *T. occidentalis* (60.3 %); the best termite mortalities were calculated for Azohoue2 (63.9 %) and Ayogbe1 (62.1 %) regardless of EPN concentration. Moreover, the Probit regressions revealed significant differences in LC_{50} among EPN isolates for both termite species (Table 5.5). The highest LC_{50} were recorded for *M. bellicosus* with *Steinernema* sp. Bembereke (23 IJ) and for *T. occidentalis* with *H. indica* Ayogbe1 and *H. sonorensis* Ze3 (19 IJ); the lowest values were recorded for *M. bellicosus* with *H. indica* Ayogbe1 and *H. sonorensis* Ze3 (11 IJ) and for *T. occidentalis* with *Steinernema* sp. Bembereke (12 IJ).

Table 5.5 Mean numbers of infective juveniles of four entomopathogenic nematode isolates required to cause 50% (LC_{50}) mortality to workers of two termite species, *Macrotermes bellicosus* and *Trinervitermes occidentalis*, within 48 h.

Termite species	EPN species	Isolates	LC_{50} [a]	95% CL [b]	χ^{2c}	Probit model	
						Intercept	Slope
M. bellicosus							
	H. indica	Ayogbe1	11 b	7-15	6.204	-2.386	2.329
	H. sonorensis	Azohoue2	13 ab	11-15	5.085	-2.453	2.196
	H. sonorensis	Ze3	11 b	7-14	5.631	-2.384	2.333
	Steinernema sp.	Bembereke	23 a	20-27	0.767	-2.485	1.827
T. occidentalis							
	H. indica	Ayogbe1	19 a	17-21	2.420	-3.953	3.124
	H. sonorensis	Azohoue2	13 ab	10-17	6.197	-2.356	2.933
	H. sonorensis	Ze3	19 a	17-22	3.821	-2.356	1.853
	Steinernema sp.	Bembereke	12 b	10-13	2.866	-2.744	2.567

[a] Concentration (number of IJ per insect) required killing 50% of treated insects; LC_{50} values within a column followed by the same letter are not significantly different, based on non-overlapping 95% CL.

[b] 95% confident limits (CL) for the LC_{50}

[c] χ^2, calculated values of chi-square;

5.3.3 Nematode repellent effect and termite ability to disperse nematodes

In the control as well as in other treatments, termite workers travelled from the A boxes into the B boxes and back within 4 h, and remained evenly distributed for the rest of the experimental period.

Mortality of larvae of *G. mellonella* due to EPN isolates was observed in A boxes indicating that nematodes were carried by termites from the B boxes into the A boxes. However, no significant difference in mortality of *G. mellonella* larvae was observed among isolates neither for *M. bellicosus* at 4 ($F = 1.13$; df = 3, 20; $P = 0.3590$) and 11 ($F = 1.47$; df = 3, 20; $P = 0.2530$) nor for *T. occidentalis* at 7 ($F = 0.67$; df = 3, 20; $P = 0.5823$) and 14 ($F = 0.15$; df = 3, 20; $P = 0.9217$) baiting days (Figure 5.3). Larval mortalities of *G. mellonella* ranged between 26.7-43.3 % and 83.3-93.3 % at 4 and 11 baiting days, respectively for *M. bellicosus*, whereas for *T. occidentalis*, they varied between 20-23.3 % and 56.7-63.3 % at 7 and 14 baiting days, respectively. In all cases, larval mortalities were higher at the second baiting time than at the first (Figure 3.3).

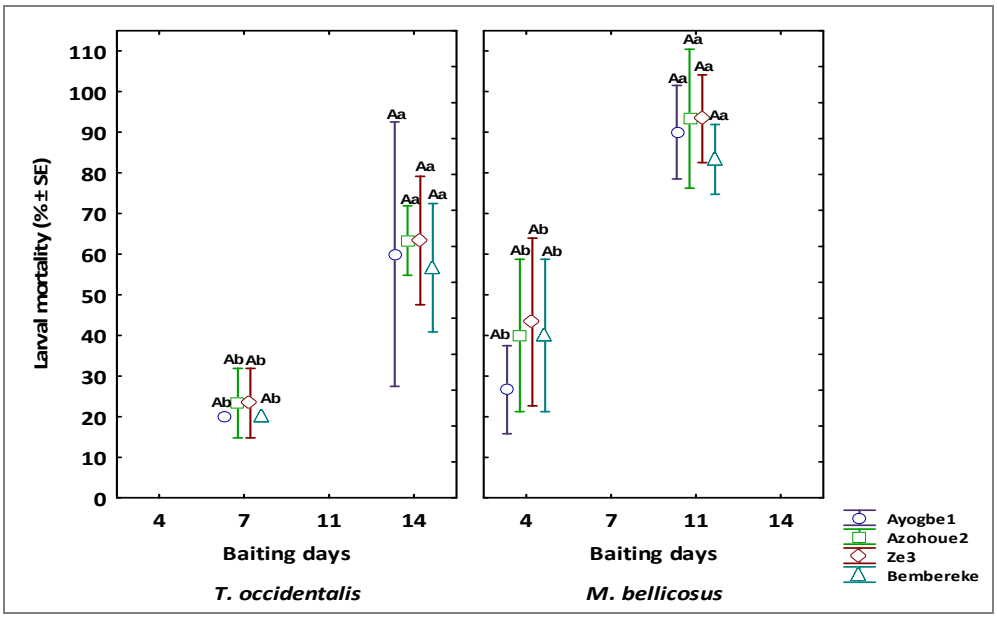

Figure 5.3 Mortality (means ± SE) of *Galleria mellonella* larvae exposed to soil in A boxes, 7-14 days (*Trinervitermes occidentalis*) or 4-7 days (*Macrotermes bellicosus*) after application of termites and nematodes (Ayobe1, Azohoue2, Ze3 and Bembereke) into A and B boxes, respectively. Boxes A and B were filled with 40 cm^3 sterilized (80°C, 72 h) sand (< 2mm) adjusted to 10% moisture, and connected at their bases by a 15 cm-transparent tubing. Larval mortality was recorded 72 h after baiting. Different capital letters on bars indicate significant differences among nematode isolates at a given baiting time; different lowercase letters on bars indicate statistical differences between baiting time for the same isolate ($P<0.05$, SNK's test).

5.4 DISCUSSION

The laboratory bioassays clearly demonstrated all four selected EPN isolates to be capable of infecting and killing the termite species *M. bellicosus* and *T. occidentalis.* This confirms reports of several previous studies demonstrating the efficacy of EPN as antagonists to termites (*e.g.* Yu *et al.*, 2010; Manzoor, 2012).

Both the nematode species and the caste significantly influenced a series of stages describing the interaction of EPN and termites; *viz.* virulence, penetration rate, and number of emerging IJ from termites. With respect to virulence of the EPN isolates, soldiers of both *M. bellicosus* and *T. occidentalis* were similarly susceptible, but more susceptible to EPN than workers. Unlike soldiers, workers of both termite species expressed differential susceptibility to the tested nematodes. This supports the findings of Mankowski *et al.* (2005) who showed that soldiers of *Captotermes formosanus* and *C. vastator* were more susceptible to *S. carpocapsae* and *H. indica* than workers. Depending on the isolate, the mortality of workers of *M. bellicosus* or *T. occidentalis* varied between 75 - 96.3% or 75 - 98.5%, respectively. In addition, the Ze3 isolate of *H. sonorensis* caused much higher mortality to both *M. bellicosus* and *T. occidentalis* than the Azohoue2 isolate of the same species, proving that different isolates of the same nematode species can differ in virulence against a certain pest (Hazir *et al*., 2001).

According to Pervez *et al*. (2012), the rate of penetration of EPN into the host insect can be used as a measure of host susceptibility. It has been proposed as an alternative to LC_{50} as an assessment of EPN efficacy (Hominick & Reid, 1990; Glazer, 1992). My experiments showed that soldiers of *M. bellicosus* (13.2 - 18.6% IJ penetration) were the most susceptible followed by workers of the same species (5.2 - 8.8% IJ penetration) compared to soldiers (1 - 1.4% IJ penetration) and workers (1.4 - 2% IJ penetration) of *T. occidentalis.* Westerman (1998) reported that host size influenced the ease with which the nematodes can penetrate into host. Both, soldiers (86 ± 6 mg) and workers (38 ± 3 mg) of *M. bellicosus* have a much larger surface area than soldiers (2 ± 0.2 mg) and workers (10 ± 2 mg) of *T. occidentalis*. This feature allows the EPN to penetrate *M. bellicosus* more easily through natural openings (mouth, anus, spiracles) or, in case of heterorhabditids, by disrupting the soft cuticle of the host by its dorsal tooth (Bedding & Molyneux, 1982). I observed that almost all IJ that had penetrated soldiers of *M. bellicosus* in particular were located in the head capsule, suggesting that oral openings may be important portals of entry (Wang & Gaugler, 1998).

In my study, the LC_{50} value obtained for each of the EPN species, supplemented the virulence data and facilitated discriminating EPN species/isolates as a function of the target termite species. Workers of *M. bellicosus* were more susceptible to *H. indica* Ayogbe1 (LC_{50} = 11 IJ) and *H. sonorensis* Ze3 (LC_{50} = 11 IJ) than the other EPN isolates, whereas workers of *T. occidentalis* were more susceptible to *Steinernema* sp. Bembereke (LC_{50} = 12 IJ) and *H. sonorensis* Azohoue2 (LC_{50} = 13 IJ). However, none of these isolates showed the highest penetration rate. Obviously, this finding does not support the assumption that nematodes demonstrating the greatest efficacy against a target insect would have the highest invasion efficiency.

Multiplication is an essential character for EPN populations to increase their chance for getting established in the insect environment (Phan *et al.,* 2005). All tested EPN isolates reproduced well in *M. bellicosus* and *T. occidentalis*; however, the level of the progeny production varied significantly between termite species/castes. The study revealed that *H. sonorensis* Ze3 was more reproductive than *H. indica* Ayogbe1, *H. sonorensis* Azohoue2 and *Steinernema* sp. Bembereke in the same termite species. This corroborates findings in , previous assays (Chapter 4) showing that the reproduction potential in *M. bellicosus* decline from *H. sonorensis* Ze3 over *H. indica* Ayogbe1 to *H. sonorensis* Azohoue2. The heaviest and largest caste termites, *viz.* soldiers and workers of *M. bellicosus* allowed the highest number of IJ per milligram insect to be multiplied whereas the small soldiers and workers of *T. occidentalis* produced the least number of IJ per milligram insect, regardless of the EPN isolates/species. This is in agreement with findings of Blinova and Ivanova (1987) and Flanders *et al*. (1996) who speculated that IJ yield is proportional to host size. Selvan *et al*. (2010) suggested that one of the criteria for determining host suitability is the level of IJ reproduction following infection. Consequently, the most suitable host for all tested EPN isolates/species was *M. bellicosus*. Further field investigations may lead to a promising biocontrol of this citrus pest in south Benin.

My EPN repellent-dispersing assay did not show evidence that *M. bellicosus* and *T. occidentalis* would be able to detect the presence of IJ of any EPN isolates/species. In a similar study undertaken by Wang *et al*. (2002), it was shown that *H. indica* (362 IJ cm^{-3}) repelled *R. flavipes* up to 17 days. In our study, however, the isolate *H. indica* Ayogbe1 did not repel either *M. bellicosus* or *T. occidentalis* at 962.5 IJ cm^{-3}. Nevertheless, the presence of EPN in box A revealed that termite workers may serve as a means of transport for EPN. Because the workers feed the other castes, enlarge and repair the nest, make tunnels, and look

after the queen, the eggs and young nymphs (Noirot & Darlington, 2000), their ability to disperse nematodes may reinforce nematodes to continue the infestation to termites in time and space after the initial EPN application.

In summary, this study demonstrated variations in susceptibility of *M. bellicosus* and *T. occidentalis* to tested EPN isolates/species and the potential of these nematodes to control termites. Further studies are currently underway to evaluate the EPN isolates/species in field conditions in order to provide evidence of their utility before recommending these biocontrol agents to farmers.

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6

Influence of pesticides, soil temperature and moisture on entomopathogenic nematodes from southern Benin and their potential to control underground termite nest populations

ABSTRACT

The influence of three commonly used pesticides, *viz*. fipronil (insectcide), sulphur (fungicide) and glyphosate (herbicide) on the viability and infectivity of four Beninese isolates of entomopathogenic nematode (EPN), *Heterorhabditis indica* Ayogbe1, *H. sonorensis* Azohoue2, *H. sonorensis* Ze3, and *Steinernema* sp. Bembereke, was determined. The impact of soil temperature and soil moisture on the virulence of these EPN to the termite species *Trinervitermes occidentalis* was investigated in laboratory conditions. Field trials were carried out to study the effect of EPN-infected *Galleria mellonella* larvae on underground populations of the termite *Macrotermes bellicosus*. Results showed that all tested *Heterorhabditis* species were more tolerant to glyphosate and fipronil than the *Steinernema* species. However, results obtained from the active substance sulphur demonstrated that the compatibility of EPN with pesticides is not only species specific, but also strain specific. The temperature level showed a negative impact on the virulence of the tested nematode isolates to workers of *T. occidentalis*, especially at 37°C. However, interesting results were obtained with *H. sonorensis* Azohoue2, which showed the best results as compared to *H. indica* Ayogbe1, with 63.2 versus 20.3 % termite mortality at soil temperature of 35°C. Unlike soil temperature, the increase of soil moisture to 20 % (w/w) did not negatively influence the virulence of EPN isolates to workers of *T. occidentalis.* Field trials showed that 70 days after application, the entire underground populations of 71% or 60 % treated nest were controlled by infective juveniles released by 50 2-week *H. sonorensis* Azohoue2- or *H. indica* Ayogbe1-infected *G. mellonella* larvae, respectively. These findings suggest that tested indigenous EPN can provide effective biological control of *M. bellicosus* in the field.

6.1 INTRODUCTION

The use of chemical pesticides in agriculture has several drastic negative aftermaths, *viz.* the development of pest/pathogen resistance to applied agents, the growing cost of the plant production, and the non-target negative environmental impacts (Gerhardson, 2002). Repeatedly, it has been demonstrated that biological control has the potential to be a useful alternative strategy (Atwa *et al.,* 2013). Entomopathogenic nematodes (EPN) of the families Heterorhabditidae and Steinernematidae have attracted the attention of nematologists as possible biological control agents in integrated pest management (IPM) programmes in their battle against economically important insect pests (Sturhan & Mráček, 2000). The Infected juvenile (IJ) of EPN is the only free-living stage occurring naturally in the soil. When an IJ encounters a host, it enters through body openings, such as the mouth, anus or spiracles. Some heterorhabditids are able to penetrate intersegmental membranes of insect cuticle by using an anterior tooth (Bedding & Molyneux, 1982). Once inside the host haemocoel, the IJ releases the symbiotic bacteria from its intestine. The bacteria proliferate inside the host haemolymph and also serve as food source for the nematode. The insect host dies within 24-48 h, due to septicemia. The IJ develop into adult, reproducing through one or more generations depending on conditions and food availability within the host, and when food becomes depleted IJ take up bacteria and leave the cadaver for the soil. The natural soil habitat, however, is a challenging environment for their persistence, considering a variety of biotic and abiotic stressors that may drastically reduce their longevity and infectivity. These stressors may be anthropogenic (*e.g.* pollutants such as pesticides) as well as natural (UV radiation, fluctuating soil temperature and moisture content, antagonists, etc.).

Some chemical pesticides can injure or kill EPN (Wright *et al*., 2005) or reduce their viability and infectivity (Hara & Kaya, 1983; Zimmerman & Cranshaw 1990; Krishnayya & Grewal 2002). However, the susceptibility of IJ to synthetic chemicals depends on several factors such as nematode species and strain, agrochemical formulation and application dose (Grewal, 2002; Laznik*et al*., 2012). Some chemical pesticides are compatible with EPN and interact synergistically against insect pests (Koppenhöfer *et al*., 2000) producing higher mortality than either agent alone. But in Benin, citrus growers often rely on chemical insecticides, mainly fipronil to control termites such as *Macrotermes bellicosus* which causes extensive damage in citrus orchards. They also used chemical herbicide (glyphosate) and fungicide (sulphur) to control weeds and the citrus black spot (*Guignardia citricarpa* kiely) fungi respectively (ESCiP-Benin, 2012). To develop a successful IPM program for insects, it is important to

ascertain the degree to which these nematodes may be affected by chemical pesticides (García-del-Pino & Jové, 2005; Gutiérrez *et al*., 2008) as well as the compatibility of IJ of EPN for certain chemical pesticides (Barbara & Buss, 2005).

Environmental conditions such as soil moisture regime and temperature, can affect nematode survival in soil (Molyneux & Bedding, 1984; Molyneux, 1985; Kung *et al*., 1991). Within the life cycle of the nematode, its entry into a host and emergence from a host is temperature-dependent and may vary between species or even strains (Wouts, 1980). Low temperatures reduce nematode mobility inducing low ability to search for a host, penetrate it (Chen *et al*., 2003) and reproduce inside the host. High temperature e.g. above 40°C appear to be detrimental to EPN (Glazer, 2002). Entomopathogenic nematodes require adequate soil moisture levels for their survival, movement and pathogenicity, which may vary among nematode species and isolates (Patel *et al*., 1997; Grant & Villani, 2003). High soil moisture levels can cause oxygen depletion and restrict nematode mobility, whereas low soil moisture levels can be lethal to these entomopathogens (Koppenhöfer *et al*., 1995; Patel *et al*., 1997).

When attempting to develop an effective IPM programme for the citrus termite pest, *Macrotermes bellicosus*, in Southern Benin, several studies were carried out to evaluate the efficacy of the first recorded indigenous EPN (Chapter 2). These studies included the characterisation of biocontrol traits (Chapter 3) and the effectiveness of EPN against citrus termites (Chapter 4 & 5). The results of the preliminary laboratory assays spurred investigating field applications.

Benin's climate is hot and humid. Southern Benin is characterized by a long dry season (December - March) and a short one-month dry season usually between mid-July and mid-August. During that drought period, mean temperatures may exceptionally reach 35-40°C and the available soil moisture is below the total evapotranspiration needs of crops (Adam & Boko, 1993). Inside the nest of the termite *Macrotermes*, the temperature and humidity remain fairly constant at about 30°C and at about 90%, respectively (Korb & Linsenmair, 2000); the maintenance of a high humidity is essential for the survival of most species of termites (Lee & Wood, 1971). Therefore, to achieve successful applications of EPN to control for example *Macrotermes* species, abiotic factors such as soil moisture and temperature are critical (Kaya, 1990; Smits, 1996).

I here report my findings on the effects of some conventionally used pesticides in Beninese

citrus production on the survival and infectivity of IJ of three species of indigenous EPN, *viz*. *H. sonorensis, H. indica* and an undescribed *Steinernema* sp.. I also report on the effect of a range of soil temperature and moisture content on the virulence of these species to termites in laboratory assays. The efficacy of EPN-infected *Galleria* larvae to kill the underground termite population was also investigated in field trials. Because in preliminary assays, *M. bellicosus* did not withstand temperatures above 33°C, the soil temperature and moisture bioassays were conducted on *Trinervitermes occidentalis*, which is often found in the same field with *M. bellicosus* in southern Benin (ESCiP, 2011). Field evaluations were conducted on *M. bellicosus*.

6.2 MATERIALS AND METHODS

6.2.1 Laboratory trials

6.2.1.1 Nematodes and insects

Workers of *T. occidentalis* were collected from a termite nest in a citrus field; these nests are small (<0.5m high). The nests were broken at the top and the broken mounds were collected along with termites and put in a 1000-ml plastic box. The box was transferred to the laboratory and maintained slightly opened in the dark at 25°C and 75-80% RH for 24 h before being used in the experiments.

All nematode species/isolates involved in this study (Table 6.1) were cultured in last instar larvae of the greater wax moth *Galleria mellonella* (Kaya & Stock, 1997). Infective juveniles were used to infect the *G. mellonella* larvae instar, which were then incubated at 27°C for 72 h. The cadavers were transferred to White traps (White, 1927) and freshly immerged IJ were used for the experiment after checking their viability through their movement under a stereomicroscope. The concentrations of IJ were adjusted by volumetric dilutions in distilled water using the formula of Navon and Ascher (2000).

Table 6.1 Species and isolates of entomopathogenic nematodes from Benin used in the study.

Species	Isolates	Accession numbers	Year of isolation	Original locality	Vegetation
H. indica	Ayogbe1	KF723816	2011	Ayogbe, Zou	Mango
H. sonorensis	Azohoue2	KF723809	2010	Azohoue-Azongo, Atlantique	Lemon
H. sonorensis	Ze3	KF723828	2011	Ze, Atlantique	Palm tree
Steinernema sp.	Bembereke	-	2012	Bembereke, Borgou	Forest

6.2.1.2 Pesticides

The effects of the three most commonly used pesticides (Table 6.2) in citrus orchards in southern Benin on EPN isolates/species were tested. Glyphosate (Glycel) is a herbicide that inhibits EPSP synthase enzyme, leading to depletion of key amino acids that are necessary for protein synthesis. Fipronil (Hercules) is an insecticide that disrups the normal function of the central nervous system in insects. Sulphur is a fungicide that forms a protective barrier on the plant surface. This barrier kills fungi by interfering with cellular respiration, inhibiting the electron movement within the fungi and preventing it from accumulating the materials and energy it needs to survive and thrive. The protective barrier also acts as a stomach toxin for the control of mites.

Table 6.2 Pesticides used for testing survival and infectivity of entomopathogenic nematodes

Trade name (Formulation)*	Pesticide classification	Active substance	Content of active substance	Field-recommended concentration
Hercules (SC)	Insecticide	Fipronil	50 g.l-1	5 ml.l-1
Sulfa (WDG)	Fungicide	Sulphur	80%	3.33 $g.l^{-1}$
Glycel (SL)	Herbicide	Glyphosate	41%	3-5 l.ha-1 in 500 l

* SC= suspension concentrate, WDG= water dispersible granule, SL= soluble liquid

6.2.1.3 Bioassay to evaluate effect of EPN exposure to pesticides

The effects of aqueous solutions of the pesticides on the IJ were evaluated in 9 cm-diameter Petri dishes. For each nematode isolate/species, 1000 IJ were suspended in a 10-ml solution of

the selected pesticides prepared at 0.5-, 1- and 2-fold the field-recommended concentration (FRC) (see Table 6.2). Nematodes suspended in distilled water were used as a control. The Petri dishes were incubated at 25°C in the dark for 24, 48, 72 or 96 h. Treatments were arranged in a split-split plot design with EPN isolates as main factor, exposure time as sub-factor and pesticide concentration as sub-sub-factor. There were four replicates per treatment (Isolates x Times x Concentrations) and the experiment was performed twice.

To assess survival, nematode suspension was first thoroughly homogenized and five 100-µl sub-samples were collected from each Petri dish at each exposure time. The IJ in the sub-samples were rinsed three times to remove pesticide before being kept in distilled water for an additional 24 h. Then nematode suspensions were transferred and stirred in a watch glass to mechanically stimulate IJ. Live and dead IJ were counted under a stereomicroscope. Nematodes that did not move, even after probing, were considered dead. Nematode mortality percentages were then calculated.

Since Fipronil is the mostly used insecticide to control termites in citrus in Bénin, therefore beyond the study of its compatibility with Beninese EPN, its effect on IJ infectivity was investigated for the development of a cost-effective integrated termite management with the combination fipronil-nematode in tank-mixes. To evaluate the sub-lethal effect of fipronil on nematode infectivity, 50 alive IJ suspended in 80 µl of the above washed nematodes-pesticide suspension, were pipetted into 2-ml Eppendorf tubes before it was filled with 1 ml of pasteurized air-dried sand (< 2 mm). In preliminary observations, a volume of 80 µl was found to provide sufficient moisture for optimal nematode activitiy including infectivity. Thereafter, one single last instar *G. mellonella* was transferred into each tube. The same procedure was implemented in the control treatment (distilled/tap water without nematodes). Each tube was then sealed with its holed lid (approx. 0.6 mm diam.) to allow air exchange. The tubes were arranged in a completely randomized design and kept in the dark at room temperature (25°C). Four replicates were used per nematode isolate with ten Eppendorf tubes per replicate. Insect mortality was recorded 72 h after inoculation. Dead insects were dissected to ascertain their infection by nematodes. The experiment was performed twice.

6.2.1.4 Effect of soil temperature on virulence to **Trinervitermes occidentalis**

The experiments evaluating the effect of soil temperature were conducted in plastic boxes (6 and 8 cm in diameter at the base and top, respectively; 4 cm high). The boxes were filled with 40 cm^3 sterilized (80°C, 72 h) sand (< 2-mm) adjusted to 10% moisture content (w/w). The surface soil was 38.5 cm^2. Temperatures tested were 27, 30, 33, 35 and 37°C over three different concentrations of IJ, *viz.* 200, 400 and 800 IJ/box (corresponding with densities of 5, 10; 20 IJ cm^{-2}). The assigned IJ concentration suspended in 60 µl of distilled water was applied in a 1-cm deep cavity made at the centre of the soil surface. The cavity was immediately covered with the surrounding soil. Afterwards, the boxes were kept in incubator to the designated experimental temperature for 1 h before receiving each 10 workers of *T. occidentalis*. Boxes without IJ served as the control to check the mortality of termites. There were four replicates per treatment (Temperature x Concentration x Isolate). A split-split-plot design with temperature as main factor, concentration as sub-factor, and EPN isolates as sub-sub-factor was used. After 72 h exposure, dead insects in individual boxes were recorded. They were rinsed in distilled water, incubated for 24 h at 25°C before being dissected to confirm insect infection by nematodes. The experiment was performed twice in the same conditions with different batches of nematodes.

6.2.1.5 Effect of soil moisture on virulence to **Trinervitermes occidentalis**

The experimental scheme that was used for testing the effect of temperature on the infectivity of EPN isolates was also used in the soil moisture experiments. Three IJ concentrations, *viz.* 200, 400 and 800 IJ/box (corresponding with densities of 5, 10; 20 IJ cm^{-2}), were used. The assigned nematode concentration suspended in 60 µl distilled water was placed at the bottom of each box which was then filled with 40 cm^3 of sterilized (80°C, 72 h) soil (< 2 mm) previously adjusted to 5, 10, 15 or 20% moisture content (w/w). Subsequently, ten workers of *T. occidentalis* were introduced in individual boxes, which were immediately covered. The boxes were placed in a plastic bag to avoid evaporation, and incubated in the dark at 27°C. Boxes without IJ served as the control to check the mortality of termites. There were three replicates per treatment (Moisture x Concentration x Isolate). A split-split-plot design with moisture content as main factor, nematode concentration as sub-factor, and EPN isolate as sub-sub-factor was used. After 72 h exposure, insects were recovered and the numbers of dead insects in individual boxes were recorded. They were rinsed in distilled water, incubated for

24 h at 27°C before being dissected to confirm insect infection by nematodes. The experiment was performed twice in the same conditions with different batches of nematodes.

6.2.2 Field trial

Only isolates *H. sonorensis* Azohoue2 and *H. indica* Ayogbe1 were used in the field trials carried out in the rainy season. The field was a citrus orchard, naturally infected with the termite pest *M. bellicosus*. It was located at Zakpota (07°12.143′N, 02°14.513′E), one of the major growing areas of citrus (specifically oranges) in Benin. The soil type was sandy clay (82.5% sand, 2.3% silt, 15.3% clay, and 1.5% organic matter), pH = 6.5. No natural colonisation of the termite nests by EPN was detected after baiting (*G. mellonella*) soil samples from the nests. The site had not been treated with insecticides during the previous year.

In preliminary assays, *M. bellicosus* colonies were able to reconstruct an aboveground nest within three months after its demolition. In this respect, the aboveground nests were first demolished before a unique dose of 50 EPN-infected *G. mellonella* larvae (two-week old), regardless of the nest size, was applied over the demolished surface. The EPN-infected larvae were covered with slightly moistened soil taken from the nest. Three treatments were compared: (1) 50 *G. mellonella* larvae infected with *H. sonorensis* Azohoue2, (2) 50 *G. mellonella* larvae infected with *H. indica* Ayogbe1, and (3) untreated control. Three parameters *viz*. the nest reconstruction progress, nematode persistence in the nests, and the number of non-viable nests (with no active termite population), were recorded.

To monitor nest reconstruction progress, nests' volumes (V) were estimated before demolition and every 10 days interval after application of the EPN-infected *G. mellonella* larvae. The nests volumes were calculated using the formula to calculate the volume of a cone $V = 1/3\ \pi * R^2 * h$, where R (m) is the nest radius and h (m) the nest height.

Nematode persistence in the nest area was assessed by randomly taking soil samples composed of 3 cores (0-15 cm depth) from each treated nest 10, 20 and 70 days after EPN-infected *G. mellonella* larvae application (DAA). The three soil core samples were individually baited with 10 last instar *G. mellonella* larvae and kept at room temperature (25 ± 1°C) for one week. Then, dead larvae were recorded daily from the fifth day to the seventh. Cadavers were dissected to confirm EPN infection. We assumed that the number of infected

larvae found by sampling was related to the number of nematodes that were present in the soil (Koppenhöfer *et al*., 1996; Susurluk & Ehlers, 2008).

Treated and non-treated nests were excavated 70 DAA and the percentage of nests for which the underground populations died was estimated. The experiment was performed twice at different times (June to September and August to December) in the same area but in different citrus orchards. In total, there were 14 replicates for treatment 1, 10 for treatment 2, and 12 for treatment 3.

6.2.3 Statistical analyses

Prior to analysis, all data were corrected for the mortality rate of the control group (< 10%) using Abbott's formula (Abbott, 1925). Because data from repeated bioassays were not significantly different, they were pooled for analysis. General Linear Model (GLM) for normal distribution with identity link function was used to analyse data acquired from testing the effect of pesticides on the survival of EPN. To stabilize the variance of means, mortality percentages were arcsine transformed and subjected to ANOVA (POC GLM, SAS V9.2). Student-Newman-Keuls (SNK) test at $P< 0.05$ was carried out to assess differences among nematode isolates. Non-transformed means are presented in figures. Linear regression analyses were performed using the REG procedure of SAS. Virulence assay data were used to calculate the survival time (ST_{50}) values using Probit analysis in SPSS V16.0 (2007) for Windows. Differences among isolates were considered to be significant when the 95% confidence limits of ST_{50} values failed to overlap.

6.3. RESULTS

6.3.1 Laboratory trials

6.3.1.1 Bioassay to evaluate effect of EPN exposure to pesticides

The general linear model revealed that the survival of IJ differed significantly among isolates, times, concentrations and pesticides (Table 6.3). The two-way interactions Isolates x Times, Isolates x Pesticides and Concentration x Pesticide were significant. However, the interaction Isolates x Concentrations was not significant.

In order to evaluate the influence of exposure time on the viability of IJ, the times (ST_{50}) at which IJ survival fall to 50% were calculated at field recommanded concentration for each tested pesticides.

Table 6.3 Results of GLM for normal distribution with identity link function, used to analyse the influence of pesticides on the survival of entomopathogenic nematodes**.**

Source of variation	df	Chi-Square	P
Isolates	3	18.4721	0.000351***
Times	3	100.8672	0.000000***
Concentrations	2	101.1652	0.000000***
Pesticides	2	39.1716	0.000000***
Isolates x Times	9	21.4842	0.010666**
Isolates x Concentrations	6	6.9785	0.322844
Times x Concentrations	6	3.1691	0.787345
Isolates x Pesticides	6	242.1235	0.000000***
Times x Pesticides	6	12.2364	0.056897
Concentration x Pesticide	4	48.0398	0.000000***

***=significant at P<0.001. ** = significant at P<0.05

Based on non-overlapping 95% confidence limits of the ST_{50} (Table 6.4), the influence of all tested pesticides on IJ differed significantly among isolates. *Steinernema* sp. Bembereke (ST_{50} = 3.3 h) was more affected by glyphosate than the isolates *H. indica* Ayogbe1, *H. sonorensis* Azohoue2 and *H. sonorensis* Ze3 which were equally affected with ST_{50} values of 7.3 h, 10.5 h and 8.7 h, respectively. The isolate *H. indica* Ayogbe1 was the least affected by fipronil with ST_{50}= 10.4 h, whereas the least affected isolates by sulphur were *Steinernema* sp. Bembereke and *H. sonorensis* Azohoue2, with ST_{50} values of 7.4 and 6.5 h, respectively.

In general, increase in exposure times and fipronil concentrations had a negative impact on the infectivity of isolates as indicated by negative slopes of regression equations: $CM = \alpha + \beta E + \gamma P$, where CM= corrected mortality (%), E= exposure time (h) and P= fipronil concentration, though not significant in all cases (Table 6.5). However, the infectivity of *H. indica* Ayogbe1 was only affected by exposure time, but not by pesticide concentration.

Table 6.4 Survival time (ST_{50}) at which survival of infective juvenile of different isolates/species of EPN from Benin fall to 50% after exposure to pesticides at field-recommended concentration over a time range of 24, 48, 72 and 96 h.

EPN isolates/species	**ST_{50}[a]** (95% CL)[b]		
	Glyphosate	Fipronil	Sulphur
H. indica Ayogbe1	7.3 a (4.8-22.4)	10.4 a (6.3-43.1)	2.3 b (0.3-2.7)
H. sonorensis Azohoue2	10.5 a (6.6-36.1)	5.1 ab (4.5-7.3)	6.5 a (4.8-12.6)
H. sonorensis Ze3	8.7 a (5.2-45.6)	3.2 abc (2.2-11.7)	2.5 b (2.2-2.8)
Steinernema sp Bembereke	3.3 b (2.7-4.5)	3 c (2.7-3.5)	7.4 a (5.5-14.2)

[a] Time (expressed in hours) at which survival fall to 50%. ST_{50} values within a column followed by the same letter are not significantly different; based on non-overlapping 95% confident limits (CL).
[b] 95% CL for the ST_{50}.

Table 6.5 Regression coefficients of the effect of pesticide at 0.5, 1 and 2 folds recommended dose and exposure times (24, 48, 72 and 96 h) on the efficacy of four EPN isolates against *Galleria mellonella* larvae for the linear regression equation: $Y = \alpha + \beta E + \gamma P$, where Y= Insect mortality (%), E=exposure time and P= pesticide concentration.

EPN isolates	α^a	β^b	γ^c	P_β	P_γ	R^2
H. indica Ayogbe1	309.3±32.6	-11±1.9	8.8±2.5	0.0002	0.0073	0.20
H. sonorensis Azohoue2	2249.3±448.6	-11±2.5	-10±3.5	0.0019	0.0186	0.32
Steinernema sp Bembereke	1509.3±414.3	-12.7±2.4	-1.3±3.2	0.0004	0.7071	0.45
H. sonorensis Ze3	2080.7±635.6	-12.3±3.6	-7.5±4.9	0.0077	0.1636	0.19

The test for the null hypothesis that $\beta = 0$ or $\gamma = 0$ is indicated by their respective P-value, i.e. P_β and P_γ and R^2 shows regression coefficient for the model.
[a] intercept ± standard error
[b] slope of exposure time ± standard error
[c] slope of pesticide concentration ± standard error

6.3.1.2 Effect of soil temperature on virulence of EPN to **Trinervitermes occidentalis**

Multifactor analysis of variance revealed that termite mortality differed significantly among EPN isolates, soil temperatures and IJ concentrations (Table 6.6). The efficacy of the tested EPN isolates was affected by temperature as indicated by the significant effect of the two-way interactions Isolates x Temperatures (Table 6.6). However, no significant effect was observed

with the two-way interactions Isolates x Concentration and Temperatures x Concentrations. The effects of temperature on EPN isolates efficacy were therefore analysed separately.

Table 6.6 ANOVA for single and interaction effects of applying four Beninese isolates of entomopathogenic nematode species (EPN) *Heterorhabditis indica* Ayogbe1, *H. sonorensis* Azohoue2, *H. sonorensis* Ze3 and *Steinernema* sp. Bembereke at three concentrations (5, 10, 20 IJ cm-^{2}) to control workers of *Trinervitermes occidentalis* at five temperatures (27, 30, 33, 35 and 37°C). Insect mortality was recorded 72 h post infection.

Source of variations	df	f	p
Isolates	3	54.16	<0.0001***
Temperatures	4	41.29	<0.0001***
Concentrations	2	45.5	<0.0001***
Isolates x Temperatures	12	6.63	<0.0001***
Isolates x Concentrations	6	1.27	0.2712
Temperatures x Concentrations	8	1.48	0.1631
Error	419	-	-

***=significant at P<0.001

Termite mortality differed significantly among EPN isolates at 27°C (F = 30.74; df = 3, 92; P < 0.0001), 30°C (F = 8.93; df = 3, 92; P < 0.0001) and 33°C (F = 7.35; df = 3, 92; P = 0.0002) and 35°C (F = 22.53; df = 3, 92; P < 0.0001) but not at 37°C (F = 1.84; df = 3, 92; P = 0.1462) (Fig. 6.1). The mortalities induced by the isolates *H. sonorensis* Ze3, *H. indica* Ayogbe1 and *Steinernema* sp. Bembereke did not differ significantly at 27°C (45.3, 35.7 and 29.1%; respectively), 30°C (51.8, 31.3 and 30.8%; respectively), 33°C (45.5, 23.7 and 26.8%; respectively), and 35°C (15, 20.3, and 19.8%, respectively). The greatest mortalities were caused by *H. sonorensis* Azohoue2 at 27°C (91.1%), 30°C (69.5%), 33°C (67.1%) and 35°C (63.2%), while the lowest values were obtained at 37°C for all tested EPN isolates/species. (Figure 6.1).

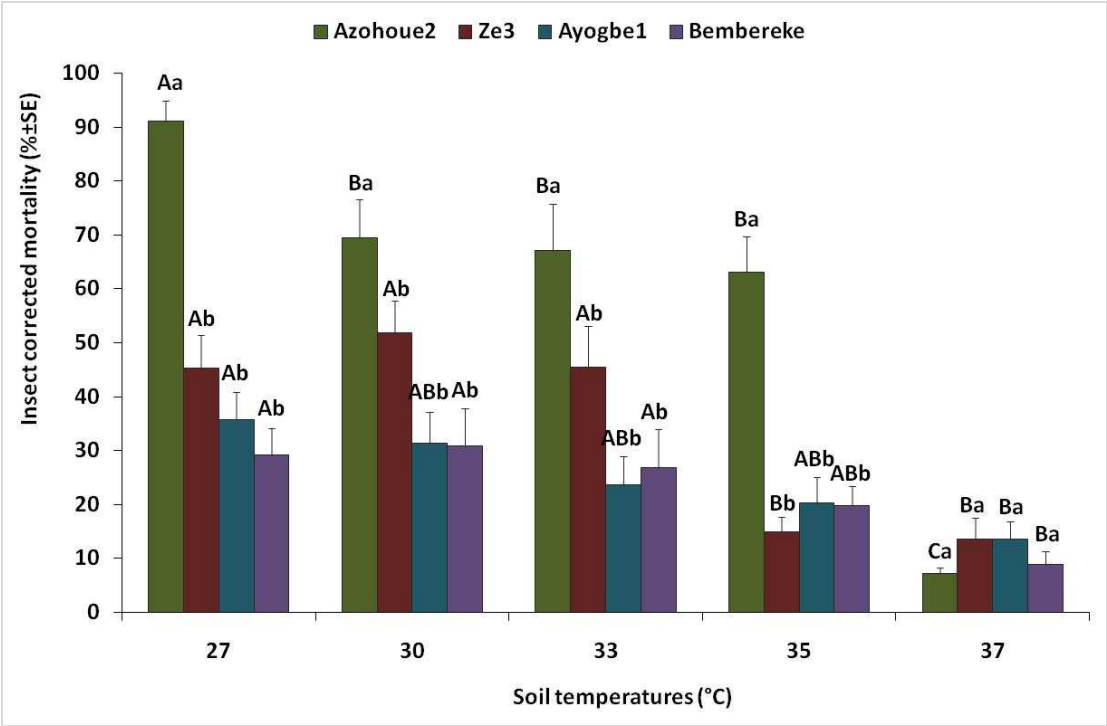

Figure 6.1 Influence of soil temperature on the virulence of the entomopathogenic nematode isolates *Heterorhabditis sonorensis* Azohoue2, *H. sonorensis* Ze3, *H. indica* Ayogbe1 and *Steinernema* sp. Bembereke from Benin to workers of *Trinervitermes occidentalis* after 72 h post-exposure (mean of all tested concentrations). Bars headed by different capital letters denote significant differences among soil temperature levels at the same EPN isolates. Bars headed by different small letters denote significant differences among EPN isolates in termite mortality at the same soil temperature (SNK's test at $P < 0.05$).

Using the forward stepwise regression procedure, the influence of fipronil concentration and soil temperature on efficacy of EPN isolates/species to workers of *T. occidentalis* could be expressed by the linear regression equation: $CM = \alpha + \beta C + \gamma T$, where CM= corrected mortality (%), C= concentration (IJ/cm^2) and T= temperature (°C) (Table 6.7). It resulted in that efficacy for all tested EPN isolates/species was influenced by both concentration and temperature with significance $0.00000 < P_\beta < 0.00003$ and $0.00000 < P\gamma < 0.00277$, respectively, and lower correlation ($0.19 < R^2 < 0.45$). However, temperature affected negatively the efficacy of all tested isolates as indicated by the negative slope γ of the linear regression equation.

6.3.1.3 Effect of soil moisture on virulence of EPN to **Trinervitermes occidentalis**

The results of the effects of soil moisture and IJ concentration on efficacy of EPN isolates are presented in Table 6.8 Termite mortality differed significantly among isolates, concentrations

and moistures. The two-way interactions Isolates x Moisture and Isolates x Concentration were also significantly different. However, the interaction Isolates x Moisture was more significant than the interaction Isolates x Concentration. ANOVA Analysis combining all tested concentrations showed the influence of soil moisture on the EPN isolates (Figure 6.2).

Table 6.7 Regression coefficients of the effect of five temperature regimes (27, 30, 33, 35 and 37°C) and three nematode concentrations (5, 10; 20 IJ/cm^2) on the efficacy of different isolates/species of entomopathogenic nematodes from Benin against a citrus termite pest *Trinervitermes occidentalis*, after 72 h post-exposure for a linear regression equation: CM = α + βC + γT, where CM = corrected mortality (%), C = concentration (IJ/cm^2) and T= temperature (°C).

EPN isolates	α^a	β^b	γ^c	P_β	P_γ	R^2
H. indica Ayogbe1	80.2±19.5	0.03±0.01	-2.2±0.6	0.0001	0.0003	0.20
H. sonorensis Ze3	135.2±22.4	0.05±0.01	-3.8±0.7	0.0000	0.0000	0.32
H. sonorensis Azohoue2	250.9±26.1	0.05±0.01	-6.7±0.8	0.0000	0.0000	0.45
Steinernema sp. Bembereke	67.5±21.1	0.04±0.01	-1.9±0.6	0.0000	0.0028	0.19

The test for the null hypothesis that $\beta = 0$ or $\gamma = 0$ is indicated by their respective P-value, i.e. P_β and P_γ and R^2 shows regression coefficient for the model

[a] intercept ± standard error

[b] slope of nematode concentration ± standard error

[c] slope of soil temperature ± standard error

Table 6.8 Single and interaction effects of applying four Beninese isolates of entomopathogenic nematodes *Heterorhabditis indica* Ayogbe1, *H. sonorensis* Azohoue2, *H. sonorensis* Ze3 and *Steinernema* sp. Bembereke at three concentrations (5, 10 and 20 IJ cm^{-2}) to control workers of *Trinervitermes occidentalis* at four soil moisture levels (5, 10, 15 and 20%, w/w). Insect mortality was recorded 72 h post infection at 25°C.

Source of variations	df	F	P
Isolates	3	8.89	<0.0001***
Moistures	3	118.13	<0.0001***
Concentrations	2	14.60	<0.0001***
Isolates x Moistures	9	3.69	<0.0002***
Isolates x Concentrations	6	2.18	0.0456*
Moisture x Concentration	6	1.33	0.2442
Error	240	-	-

*=significant at P<0.05; *=significant at P<0.0001

EPN isolates differed significantly in mortality induced to *T. occidentalis* at 5% (F = 4.17; df = 3, 68; P = 0.0091), 10% (F = 3.01; df = 3, 68; P = 0.0359), 15% (F = 4.99; df = 3, 68; P = 0.0035) and 20% (F = 4.82; df = 3, 68; P = 0.0042) soil moisture content. The lowest mortality was obtained at 5% moisture for all tested isolates (Figure 6.2). None of the isolates, except *H. sonorensis* Ze3, caused significant differences in termite mortality over soil moisture range of 10%, 15% and 20%. Termite mortalities ranged between 64% and 70% for *H. indica* Ayogbe1, 81% and 84% for *H. sonorensis* Azohoue2 and between 68% and 90% for *Steinernema* sp. Bembereke. The isolate *H. sonorensis* Ze3 caused a lower mortality to *T. occidentalis* at 10% (68%) than at 15% (90%) and 20% (90%) soil moistures (Figure 6.2).

Forward stepwise regression procedure yielded a linear regression equation: $CM = \alpha + \beta C + \gamma M$, where CM= corrected mortality (%), C= concentration (IJ/cm^2) and M= soil moisture (%, w/w) (Table 6.9). EPN virulence was not badly affected either by IJ concentration or by soil moistures as indicated by the positive values of the standardized coefficient β and γ.

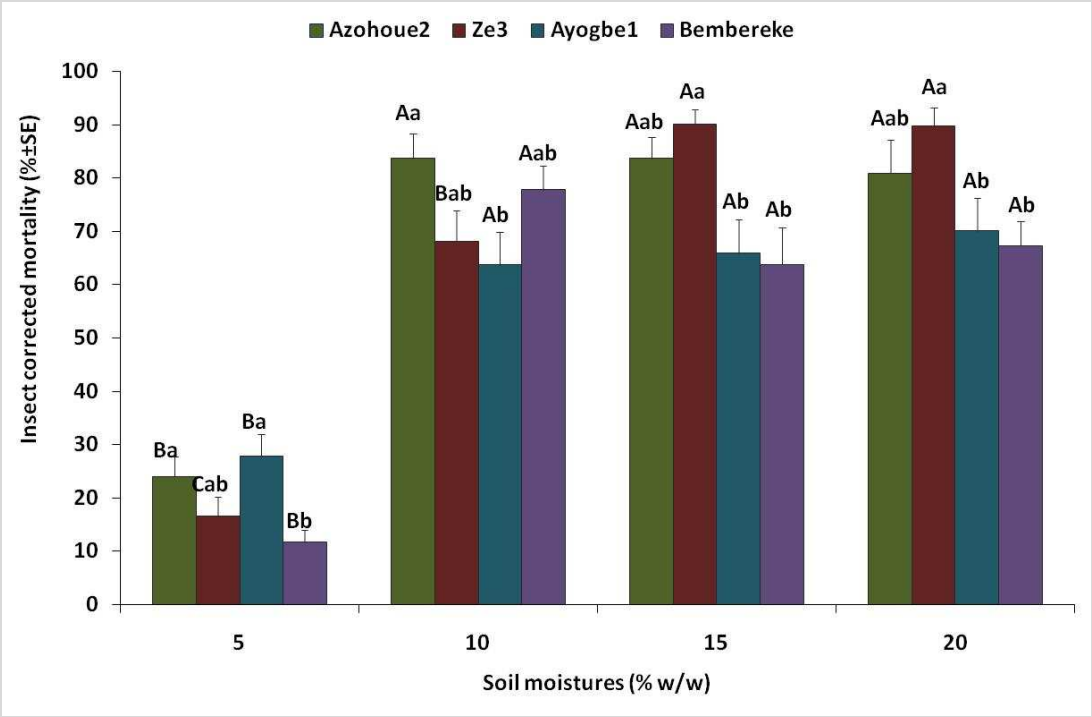

Figure 6.2 Influence of soil moisture on the virulence of the entomopathogenic nematode isolates *Heterorhabditis sonorensis* Azohoue2, *H. sonorensis* Ze3, *H. indica* Ayogbe1 and *Steinernema* sp. Bembereke from Benin to workers of *Trinervitermes occidentalis* after 72 h post-exposure (mean of all tested concentrations). Bars headed by different capital letters denote significant differences among soil moisture levels at the same EPN isolates. Bars headed by different small letters denote significant differences among EPN isolates in termite mortality at the same soil moisture content (SNK's test at $P < 0.05$).

Table 6.9 Regression coefficients of the effect of four soil moisture levels (5, 10, 15 and 20% w/w) and three nematode concentrations (5, 10, and 20 IJs/cm^2) on the efficacy of different isolates/species of entomopathogenic nematodes from Benin against a citrus termite pest, *Trinervitermes occidentalis*, after 72 h post-exposure for a linear regression equation: CM = α + βC + γH, where CM= corrected mortality (%), C= concentration (IJ/cm^2) and H= moisture (w/w).

EPN isolates	α^a	β^b	γ^c	P_β	P_γ	R^2
H. indica Ayogbe1	6.7±8.8	0.04±0.01	258.3±51.2	0.0010	0.0000	0.35
H. sonorensis Ze3	-0.5±7.8	0.02±0.01	482.6±45	0.1810	0.0000	0.63
H. sonorensis Azohoue2	7.5±9.2	0.04±0.01	343±5.8	0.0030	0.0000	0.43
Steinernema sp Bembereke	11.7±10.2	0.01±0.01	305.8±0.6	0.4000	0.0000	0.28

The test for the null hypothesis that $\beta = 0$ or $\gamma = 0$ is indicated by their respective P-value, i.e. P_β and P_γ and R^2 shows regression coefficient for the model.

[a] intercept ± standard error

[b] slope of nematode concentration ± standard error

[c] slope of soil moisture ± standard error

6.3.2 Field trial

General linear model for normal distribution with Log link function, used to analyse the influence of EPN on *Macrotermes bellicosus* nest reconstruction following application of 50 2-week old EPN-infected *G. mellonella* larvae differed significantly among treatments (χ^2 = 0.0000; df = 2; P < 0.0000), exposure times (χ^2 = 933.7654; df = 7; P < 0.0000). The interaction treatment x exposure times also differed significantly (χ^2 = 525.0178; df = 11; P < 0.0000). The aboveground nest volume rose sharply during the first 10-DAA for all treatments (Figure 6.3). After that period, it continued to grow dramatically for treatment 3 (untreated control) until 40 DAA, whereas for treatments 1 and 2, the nest volume growth became very weak. From 40 DAA onwards, the increase of nest volume with treatment 3 continued but became strong, whereas for treatments 1 and 2 the growth stabilized and remained unchanged after 40 and 50 DAA respectively (Figure 6.3). In all cases, a significant correlation was obtained between nest volume rate and time: Treatment 1 (P<0.000, R = 0.761); Treatment 2 (P>0.000, R = 0.809) and Treatment 3 (P<0.000, R = 0.956).

Table 6.10 Results of GLM for normal distribution with Log link function, used to analyse the influence of larvae of *Galleria mellonella* infected with entomopathogenic nematodes on *Macrotermes bellicosus* nest reconstruction**.**

Source of variation	df	Chi-Square	P
Treatments	2	0.0000	0.00
Times	7	933.7654	0.00
Treatments x Times	11	525.0178	0.00

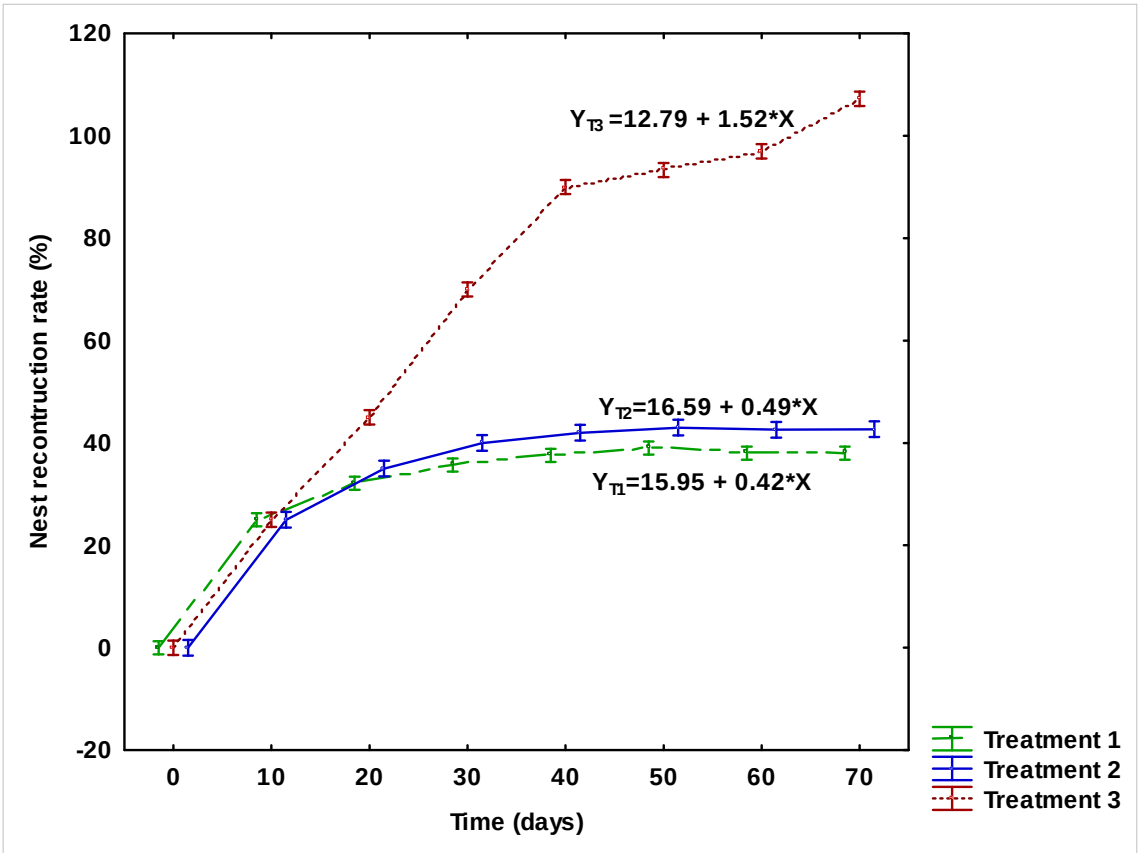

Figure 6.3 Influence of entomopathogenic nematodes on *Macrotermes bellicosus* nest reconstruction following application of 50 2-week old *Galleria mellonella* larvae infected with *Heterorhabditis sonorensis* Azohoue2 (Treatment 1) or *H. indica* Ayogbe1 (Treatment 2) or Untreated control (Treatment 3) on aboveground-demolished *Macrotermes bellicosus* nest. Vertical bars denote 0.95 confidence intervals.

Significant differences in mortality of wax moth larvae were observed among sampling times ($F = 107.85$; $df = 2, 57$; $P < 0.0001$); however, no difference was observed among treatments ($F = 0.10$; $df = 1, 57$; $P = 0.7535$). The interaction between treatments and sampling times was not significant ($F = 1.06$; $df = 2, 57$; $P = 0.3528$). The highest mortality of *G. mellonella* larvae exposed to a sample collected from treatment 1 (93.6%) or treatment 2 (95.7%) was observed at 10 DAA. In samples collected at 20 DAA, larvae mortality declined to 31.4% or 27.1%, respectively. In samples collected at 70 DAA mortality rose up to 60% or 52.8%; respectively (Figure 6.4).

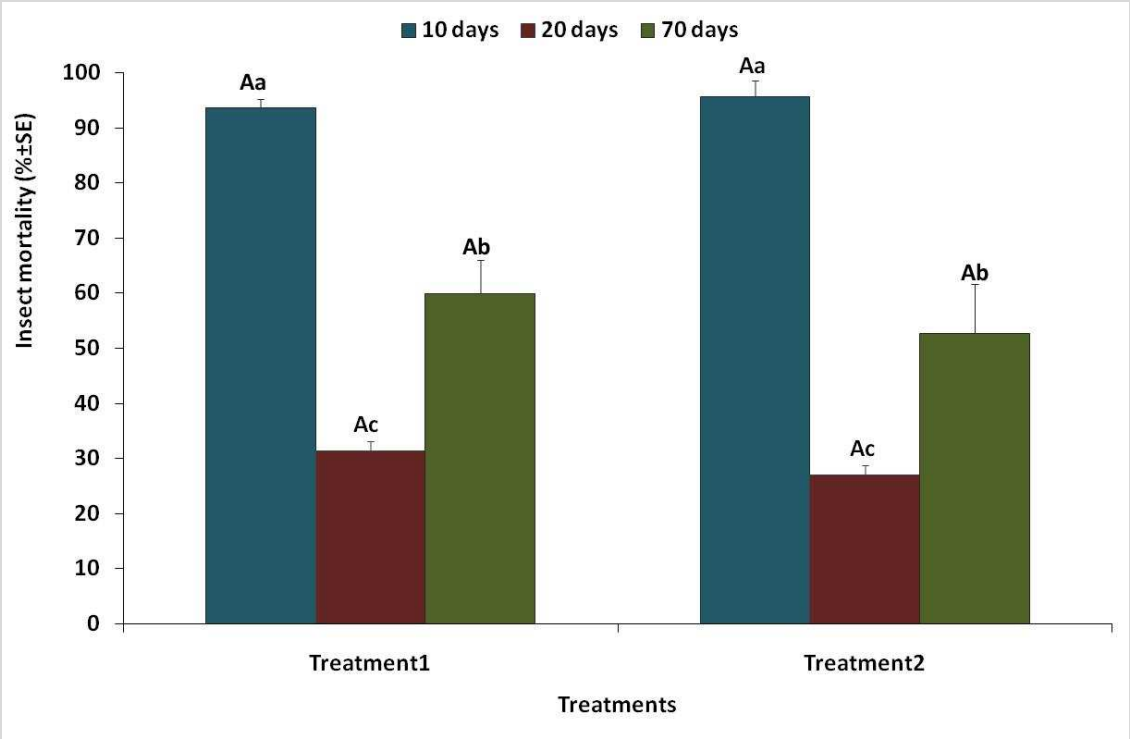

Figure 6.4 Mortality (means ± SE) of *Galleria mellonella* larvae exposed to soil samples collected 10, 20, and 70 days after application of 50 2-week old *G. mellonella* larvae infected with *Heterorhabditis sonorensis* Azohoue2 (Treatment 1) or *H. indica* Ayogbe1 (Treatment 2) on aboveground-demolished nest of *Macrotermes bellicosus*. Bars headed by the same capital letters are not significantly different among EPN isolates for a given sample collection time. Bars headed by the same small letters are not significantly different among times for a given EPN isolate (SNK's test at $P < 0.05$).

At 70 DAA, the underground populations of 71% (treatment 1) and 60 % (treatment 2) of termites were found dead after excavation, whereas the underground populations of the untreated control nests, were all alive (100%) (Figure 6.5).

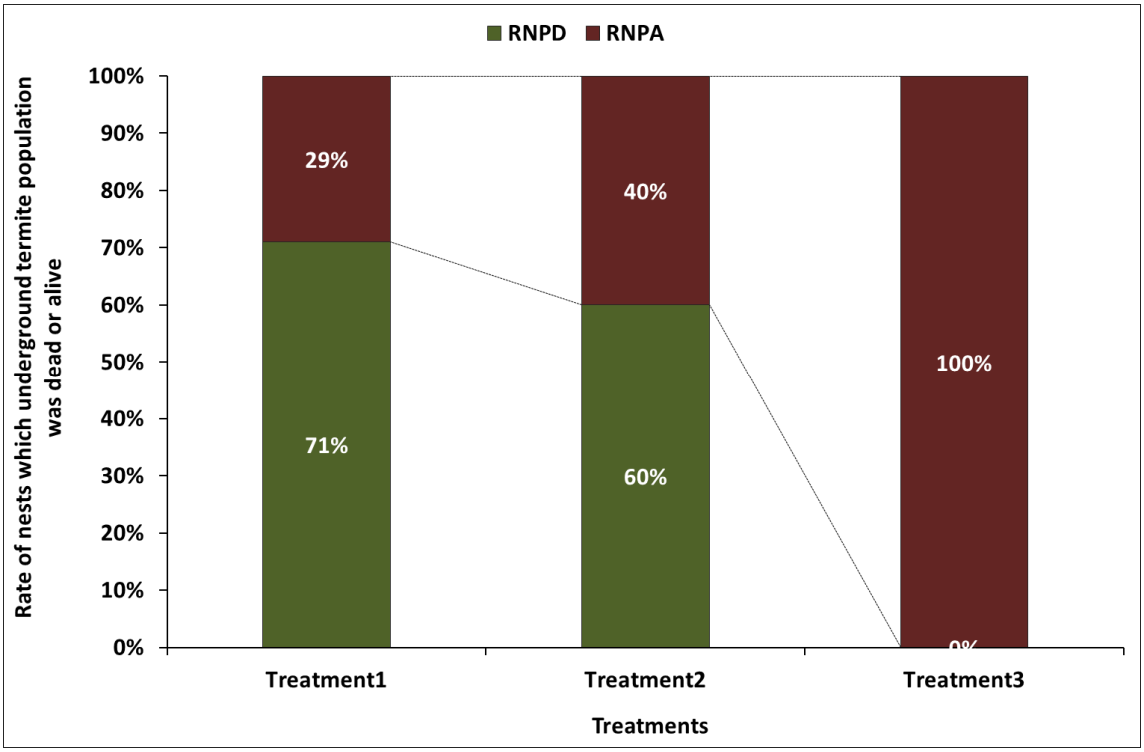

Figure 6.5 Rate of nests of which the underground termite population is controlled (RNPD) or alive (RNPA) 70 days after application of 50 2-weeks old *Galleria mellonella* larvae infected with *Heterorhabditis sonorensis* Azohoue 2 (Treatment 1) *H. indica* Ayogbe1 (Treatment 2) or untreated control (Treatment 3) on aboveground demolished nest.

6.4 DISCUSSION

Although, the effect of chemical pesticide on EPN has been investigated in many laboratory bioassays with exposure of nematodes to pesticides (De Nardo & Grewal, 2003; García-del-Pino & Jové, 2005; Laznik *et al*., 2012; Gutiérrez *et al*., 2008; Atwa *et al*., 2013), it is still necessary to test the commonly used pesticides in the area where EPN are anticipated to be used. Endemic nematode strains may differ in sensitivity to different formulations of the same pesticide (Rovesti & Deseö, 1990, Krishnayya & Grewal, 2002). Therefore, before tank-mixing newly isolated EPN with any pesticides, their compatibility should be checked. In my

study all tested *Heterorhabditis* species were more tolerant to glyphosate than the *Steinernema* species. This is different from the study of García-del-Pino and Morton (2010) in which *Steinernema* species (*S. feltiae*) was more tolerant to the enzymatic inhibitor herbicid glyphosate than *Heterorhabditis* species (*H. bacteriophora*); in the study carried out by Negrisoli Jr. *et al*. (2008), no differences were reported on the effects of glyphosate on the IJ of both nematode species. My study also showed that *Heterorhabditis* species were more tolerant to fipronil than the *Steinernema* species with *Steinernema* sp. (Bembereke) being the most sensitive to friponil and *H. sonorensis* (Azohoue2 and Ze3) the least. García-del-Pino & Jové (2005) on the contrary observed that *H. bacteriophora* and *S. carpocapsae* were similarly, highly tolerant to fipronil, whereas *S. arenarium* was more sensitive. My study includes only one isolate of *Steinernema* and so more isolates should be investigated to allow firm conclusions. It has been shown that the compatibility of EPN with pesticides is not only species specific, but also strain specific (De Nardo & Grewal, 2003; García-del-Pino & Jové, 2005; Laznik et al., 2012; Gutiérrez et al., 2008; Atwa et al., 2013) which is supported by my study. The active substance sulphur (fungicide) reduced significantly more the population density of living IJ of the isolate Ze3 than that of Azohoue2, both isolates belonging to the same species *H. sonorensis*. Moreover, *H. indica* Ayogbe1 and *H. sonorensis* Ze3 were significantly more affected by the above-mentioned fungicide than *H. sonorensis* Azohoue2 and *Steinernema* sp. Bembereke. Nermut and Mracek (2010) tested the effect of thirteen pesticides on EPN and showed that only the fungicide containing sulphur was the most toxic to all tested species of *Steinernema* including *S. feltiae*, *S. arenarium* and *S. kraussei*. In my study, however, the fungicide containing sulphur appeared to be the least toxic to the sigle species of *Steinernema* tested. Barbarossa *et al*., (1996) and Rovesti *et al*. (1988) had also observed that the fungicide containing sulphur was non-toxic to both *S. carpocapsae* and *H. bacteriophora*.

Concerning the effect of fipronil on the infectivity of Beninese EPN, my results did not match with those of García-del-Pino & Jové (2005) who showed that fipronil had negligible effects on the infectivity of all tested nematode species. In my study, both fipronil concentration and exposure time affected badly the infectivity of Beninese EPN.

Among the factors operating at the time of application or during the hours following an application of IJ or its emergence from an insect cadaver, soil temperature and moisture regime are critical for the establishment and efficacy of EPN in fields. Both factors directly influence host searching (Byers & Poinar, 1982), pathogenicity (Molyneux & Bedding, 1984;

Molyneux, 1986), and survival (Kaya, 1990; Kung & Gaugler, 1990, 1991). In my study, an increase in temperature (from 27 to 37°C) showed negative impact on the virulence of the tested EPN isolates to workers of *T. occidentalis*, the lowest mortality being observed at 37°C. *Heterorhabditis indica* is known to maintain efficacy at temperatures above 29°C (Grewal *et al.*, 1994). In my study, *H. sonorensis* Azohoue2 showed better termite mortality results compared to *H. indica* Ayogbe1 at 35° C. Unlike soil temperature, the increase of soil moisture up to 20 % (w/w) did not reduce the virulence of EPN isolates to workers of *T. occidentalis*; however, a moisture content of 5% did reduce the virulence. This suggests that lower soil moisture leads to the lack of water in the pores, limiting nematode locomotion (Glazer, 2002). However, it has been revealed that some species can develop survival strategies under water stress conditions, by reducing the body surface area exposed to the air and their cell metabolism. This process, known as anhydrobiosis, allows the nematode to overcome slow desiccation. Anhydrobiosis can be reversed when the soil become wet again, causing a recovery of nematode infectivity and virulence. On the other hand, high soil moisture levels can lead to oxygen depletion and restrict EPN's locomotion (Koppenhöfer *et al.* 1995, Patel et *al.* 1997). The EPN isolates used in this study are known to exhibit a cruiser behaviour (Chapter 4), hence optimum moisture is of paramount important to the IJ to move towards and infect a host. It has been reported that the optimum soil moisture for an effective EPN infection are within the range of 8-18% for *H. indica*, 6-20% for *S. thermophilum* and 8-25% for *S. glaseri* (Yadav & Lalramliana, 2012). This is in agreement with the optimum soil range of 10-20% revealed in this study for all tested EPN isolates.

The field trial showed that IJ that emergingfrom larvae of *G. mellonella* infected with the isolates *H. sonorensis* Azohoue2 or *H. indica* Ayogbe1, can be, to a certain extent, effective in controlling underground populations of *M. bellicosus*. In spite of the persistence of EPN in all treated nests throughout the experimental period, the failure to kill the underground population of *M. bellicosus* in certain treated nests of treatments 1 and 2 is hard to explain. It has been reported that antagonists in the field could prevent the EPN from realising their full potential as bio-insecticides (Smits, 1996; Gray, 1988) or that termites may wall-off infected-termites or groom each other to prevent infection by EPN (Fujii, 1975; Epsky & Capinera, 1988; Wang *et al.*, 2002).

Entomopathogenic nematodes and subterranean termites can thrive in similar environments. However, the control of subterranean termites has a history of limited success. One of the reasons is the very behaviour of termites such as allogrooming, walling-off, abdominal tip-

raising and self-scratching when exposed to EPN (Wilson-Rich *et al*. 2007, Wang *et al.*, 2002). Moreover, the large number of individuals in a termite colony may also be a hindrance for EPN efficacy. In that regard, an integrated subterranean termites management involving EPN that can withstand inside nest soil temperature and moisture applied sequentially or simultaneously with an insecticide may be a successful strategy to control of subterranean termites. Based on the outcome of the current study, sequential instead of simultaneous application (Head *et al*., 2000) of Beninese EPN and the insecticide fipronil might be the better choice to control *M. bellicosus* in citrus in Benin. Thus, the insecticide fipronil should be applied at the first time in order to lower the number of termites in the colony. Then the application of formulated Beninese EPN-infected *G. mellonella* larvae will follow 1-2 week later.

This study is one of a series anticipated for the implementation of a cost-effective termite management using EPN in citrus in Benin. Further investigations are currently underway to assess the efficacy of the combination of the insecticide fipronil with Beninese EPN in field conditions to provide evidence before recommending this approach to farmers.

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7

General discussion

In the framework of this PhD project, the use of native EPN to control termite pests in citrus orchards was investigated. Several species of EPN are commercially available but only 2 species of *Heterorhabditis (H. indica* and *H. bacteriophora*) and one species of *Steinernema* (*S. feltiae*) have been recorded from Africa. None of these species has been used so far to control termites in the field (Shapiro-Ilan & Gaugler, 2010). Lack of efficient transport and short shelf life of the EPN control products are a challenge under tropical climate conditions. Commercial production of EPN in large fermenters is a costly investment and requires technical skills. In developing countries commercial production of EPN is largely a cottage industry. The possible negative elements of a more labour intensive cottage industry do not apply since labour costs are low. Indigenous species of EPN are more suitable for control of local insect pests because they are better adapted to local climate and other population regulators (Bedding, 1990).

One of the first and most important needs in biocontrol programmes is the accurate identification of the pest and any beneficial organisms with biocontrol potential. This basic but indispensable information eventually impacts directly on the success of the beneficial organisms as biocontrol agents (Lacey *et al.*, 2001). In this respect, I carried out for the first time surveys for EPN in Southern Benin. Occurrence of termite pests in citrus orchards was also investigated during the surveys. Insect-baiting method using *G. mellonella* larvae was used to recover EPN from soil samples (Bedding & Akhurst, 1975). Sequence analysis was first performed and revealed all recovered EPN to belong only to the genus *Heterorhabditis*. Entomopathogenic nematodes are morphologically conservative, and molecular techniques must lead traditional morphological methods. Morphological examination was then performed to support sequence analyses. Recovered EPN isolates were then identified as *H. indica* and *H. sonorensis.*

The absence of *Steinernema* isolates suggests that one round of sampling, as pointed out by Fan & Hominick (1991), is frequently insufficient to estimate nematode diversity. In addition, repeated baiting (at least 2-3 consecutive baiting rounds), may increase the chance of recovering *Steinernema* isolates. In 2012, an extra survey was carried out in the northern part of the country. Insect (*G. mellonella* larvae) baiting method was used to collect nematodes from samples. Molecular analysis revealed the presence of 4 new undescribed species of *Steinernema*. One population of one of the new *Steinernema* species (*Steinernema* sp. Bembereke) was used in my experiments (Chapter 5 & 6).

Galleria larvae were produced in laboratory and used for bioassays and nematode regeneration. During the course of my experiments, I noticed that *Galleria mellonella* hardly reproduced during drought season period (November-March) where temperature can rise up to 40°C. This situation resulted in the lack of *Galleria* larva, compromising nematode regeneration and therefore constituting a threat for nematode maintenance. The finding of another host that can be easily produced in drought season is therefore crucial. Moreover, decrease of infectivity has been observed in some Beninese nematodes after storage for six weeks at 13-15°C. Grewal (2002) reported temperature as the most important factor affecting nematode survival. Each species requires specific optimum storage temperature, which is lower than the optimum temperature for activity and reproduction of the species and reflects the climatic conditions of its origin. Therefore investigations on optimal temperature and density are needed for long-term storage of Beninese nematode specimens.

The finding of *H. indica* in south Benin demonstrates its prevalence in tropical and subtropical zones, which is in agreement with other reports (Hominick, 2002; Khatri-Chhetri *et al*., 2011). Regarding *H. sonorensis*, the species was described for the first time from the Sonoran desert in Mexico (Stock *et al*., 2009). This is the second record and so the first for Africa. The presence of *H. sonorensis* in these two different parts of the world is probably because they both prefer a tropical climate. South Benin has a subtropical climate with two rainy seasons, whereas the Sonoran Desert has a dry tropical climate also with two rainy seasons. Moreover, in the Sonoran desert, about half of the biota is tropical in origin. Its occurrence in similar ecological areas in other surveys may strengthen our hypothesis.

The most frequent termite species found in citrus orchards during the surveys belong to the subfamily Macrotermitinae, *viz., Amitermes guineensis, Ancistrotermes crucifer, Trinervitermes occidentalis* and *Macrotermes bellicosus* (ESCiP-Benin, 2011). The latter being the most dominant and damaging, justified its choice in our study. Moreover, this species has been reported in a neighbour country, Nigeria, to be a pest of several crops including sugarcane (Boboye, 1986), maize (Wood *et al.*, 1980), groundnut (Johnson & Gumel, 1981) and cocoa (Ndubuaku & Asogwa, 2006). *Trinervitermes occidentalis* was found to occur in the same field with *M. bellicosus*; and was therefore also included in our study. Both subterranean termite species chosen have differing diets and temperature preferences. *Macrotermes* is a fungus-growing termite that prefers temperature around 30°C for optimal fungal growth all year round (Lüscher, 1961; Ruelle, 1964), whereas

Trinervitermes is a grass-eating termite whose centre mound temperatures may reach a peak of 40 °C (Adam, 1993).

The predominant method of termite control worldwide over the years has been chemical. However, chemical pesticides pose real costs to public health and to the environment (Weinzierl & Henn, 1991). The continuing pressure for regulating harmful chemical pesticides requires the development of alternative methods that emphasize least-toxic and non-chemical applications. Prospects for the development of alternative termite control using EPN need to be investigated.

In laboratory bioassays, Beninese isolates of *H. sonorensis* and *H. indica* infected and killed the target termite pest *M. bellicosus*. The majority of the isolates (73 %) killed more than 80 % of the exposed termites after three days at a concentration of 50 infective juveniles (IJ)/termite. Similarly, Georgis *et al.* (1982) recorded the infectivity of nematodes against termites in laboratory experiments. They observed 96-98% mortality of *Zootermopsis* and *Reticulitermes* with *Neoaplectana carpocapsae* (Breton strain) as well as *Heterorhabditis heliothidis*, respectively at a concentration of 200 IJ/termite, three days after application in standard Petri dishes. Wang *et al*. (2002) studied the infectivity of four nematode species, *S .carpocapsae* (Breton), *S. riobrave* (TX), *H. bacteriophora* (HP88) and *H. indica* (Coimbatore), against two termite species, *R. flavipes* and *Coptotermes formosanus* in the laboratory. They applied 400 IJ/termite and found that all nematode species were effective against *C. formosanus.* With *R. flavipes*, the results were not successful even at a rate of 2000 IJ/termite. El-Bassiouny *et al.* (2011) exposed the subterranean termites *Psammotermes hypostoma* and *Anacanthotermes ochraceus* to *H. indica* at 40 IJ/termite. They observed 23.4 and 20 % mortality after three days, increased gradually to reach 78.2 and 70 % after seven days, respectively. Knowledge on the interaction of different EPN species/isolates and different termite species could be a driving force for success in the use of EPN as biological agents against termites.

Environmental tolerance is a key character of EPN when used to control pests. The environmental characterization of EPN has been used to select better candidates for biological control (Shapiro-Ilan & McCoy, 2000; Shapiro-Ilan *et al*., 2003; Ma *et al.*, 2013). In southern Benin, vertisol and hydromorphic soils are the most dominant; the weather is tropical and temperatures may sometimes rise as high as 35 - 40°C (Faure & Volkoff, 1998). Consequently, for the EPN to be successful, it is necessary to find isolates that are able to

overcome hypoxia (lack of oxygen in vertisol), desiccation (humidity stress in hydromorphic soil) and high temperatures (warm weather). Laboratory screening of 30 Beninese EPN isolates (29 isolates of *H. sonorensis* and one of *H. indica*) showed different responses to hypoxia, desiccation and heat tolerances. As a result, six isolates of *H. sonorensis* (Zagnanado, Kpedekpo, Akohoun, Djidja2, Kassehlo and Zoundomey) proved to be the most adapted under conditions where heat tolerance is required. Nine of the *H. sonorensis* isolates (Kpanroun, Setto1, Zoundomey, Azohoue1, Azohoue2, Kemondji, Akohoun, Kassehlo and Dan) proved to be the most adapted under desiccation conditions; whereas most of them proved to tolerate hypoxic conditions. The only isolate of *H. indica* (Ayogbe1) that was detected in the surveys, showed poor heat and desiccation tolerances. *H. indica* has been considered relatively heat tolerant (Shapiro & McCoy, 2000) and so the low heat and desiccation tolerances observed are questionable. Slowing the rate of drying and rate of temperature increase might have substantial beneficial effects on long term survival of EPN infective juvenile. The bioassays did not reveal any single isolate to be superior to the others for all of the tested factors. Similar results were observed by Ma et *al.* (2013) when screening 32 different Chinese EPN isolates (belonging to 10 species) to heat, cold and desiccation tolerance. The authors found that *S. carpocapsae*, *S. ceratophorum*, *S. longicaudum*, *H. indica*, and *H. bacteriophora* proved to be more adapted to high temperatures while *H. megidis*, *H. bacteriophora*, and *S. carpocapsae* possessed better cold tolerance and *S. carpocapsae*, *S. hebeiense* and *S. ceratophorum* appeared to be better adapted to desiccation. Moreover, the isolate *H. indica* (Ayogbe1) tested in our study, and the isolate *H. indica* tested in their study tolerated heat similar when exposed at 40°C for 2 h, with 75 and 77% IJ survival; respectively. They originate from tropical and warm temperate zone, respectively. My results support the statement of Grewal *et al*. (1994) suggesting that each nematode species has a relatively well defined thermal niche breadth, which may be unaffected by their locality.

My study revealed heat, hypoxia and desiccation affect the pathogenicity of tested EPN isolates to *G. mellonella*, an insect known as the most susceptible host to infection by EPN (Woodring & Kaya, 1988). This indicates that field application of EPN suspended in aqueous solution, may directly expose them to the environmental extremes, compromising their potential as bio-pesticide. Low post-application survival in the soil reduces nematode efficacy (Smits, 1996). The development of formulations that provide protection to the nematodes from environmental extremes during and after application can substantially enhance nematode

efficacy (Grewal, 2002). Infective juveniles of EPN applied in the form of infected wax moth (*G. mellonella*) larvae (Welch & Briand, 1960; Janson & Lecrone, 1984), or their encapsulation in calcium alginate gel beads (Kaya & Nelsen, 1985), baits containing IJ, an inert carrier (e.g. corncob grits, groundnut hulls or wheat bran), a feeding stimulant (e.g. glucose, malt extract, molasses or sucrose) or a sex pheromone have been developed (Georgis, 1990) to improve nematode post application survival. It has been reported that EPN-infected *G. mellonella* larvae were as effective as aqueous nematode suspensions against soil pests (Welch & Briand, 1960; Janson & Lecrone, 1984) or can be superior to aqueous suspensions (Shapiro & Glazer, 1996).

The effectiveness of an EPN isolate largely depends on the capacity of IJ to migrate, penetrate and kill the host (Lewis, 2002). Once in the proper habitat, IJ must locate insect hosts. The foraging strategy has been used to make recommendations on which species/isolates to use in biological control programs. Thus, understanding host-finding strategies may increase my ability to make efficacy predictions, thereby optimizing host-parasite matches. Subterranean termite colonies are huge, and termite population can number in the millions, all hidden from easy view in a cryptic soil habitat (Bignell, 2011). Termite social behaviour, foraging range and habitat structure may hinder EPN efficacy. To overcome *M. bellicosus* termites in their nest, EPN isolates should be highly mobile in searching and invading their host. By doing that, they may avoid being walled-off by termites. Moreover, the grooming behaviour of termites might not prevent them realizing their duty. In my study, Beninese *H. sonorensis* and *H. indica* isolates exhibit a cruiser type of insect search strategy, corroborating that most species, including all *Heterorhabditis* spp., are regarded as cruisers (Campbell & Gaugler, 1997). Cruise-foraging EPN species are highly active and generally subterranean, moving significant distances using volatile cues to find their host underground (Gaugler *et al*., 1997). This suggests that the tested Beninese EPN isolates might reach all termite castes including the reproductive (queen, king and supplemented reproductives), the soldiers and the workers, living in the underground nest.

In general, the quantitative response of pest insects depends on the concentration of EPN isolates, as well as on the time of their exposure to their infection. The best matches tend to be for EPN that have high virulence toward their host in the protected environment (Drees *et al.*, 1992). The effect of the variation of concentration or exposure time on pest mortality is commonly used to measure the virulence of the EPN. Parametric models such as time-parasitism response (IT_{50}), dose-mortality response (LC_{50}), and time-mortality response

(LT_{50}) are useful strategies to discriminate EPN isolates for their virulence against a host (Hominick & Reid, 1990; Grewal, 2002). In my study, all tested EPN isolates appeared to have similar LC_{50} values ranging between 9 and 16 IJ/termite. 40 IJ/termite were enough to cause 80% mortality of *M. bellicosus* workers three days after application. Wang *et al*. (2002) needed much higher numbers (400 IJ/termite) to cause 58 and 73% mortality of *Reticulitermes flavipes* and *Coptotermes formosanus* after 4 days. This indicates that different host species may react in different ways when they are exposed to different EPN species.
Contrary to the LC_{50}, *M. bellicosus* exhibited time-dependent susceptibility to the tested EPN isolates. According to Hominick and Reid (1990), nematodes with the greatest efficacy against a target insect would have the highest invasion efficiency. In this respect, based on their lowest values of IT_{50} (3.35 and 3.67 h, respectively) and their highest penetration rates (11.4 and 10.0%, respectively, after 12 h exposure) the isolates Ze3 and Azohoue2 appeared the most virulent among the tested *H. sonorensis* isolates. The IT_{50} value of the *H. indica* Ayogbe1 was estimated at 5.8 h. The concentration determined in the laboratory may not be suitable under field conditions where the number of uncontrolled factors is greater. At laboratory conditions, the insect is directly exposed to the pathogen. However, under field conditions the nematode is exposed to a series of deleterious factors such as temperature and humidity, apart from having to search for the host.

Besides tolerances to environmental features, host finding strategies and virulence, multiplication is an essential character for EPN populations to increase their chance for getting established in the insect environment (Phan *et al.*, 2005). All tested EPN isolates were able to reproduce in *M. bellicosus*. When applying EPN in biological control programmes, a good multiplication inside the host insect provides high field density of IJ and may thus have long-term effects on the pest populations (Peters, 1996). My study revealed that the reproduction potential varied between 20,213 IJ/termite (*H. sonorensis* Yokon) and 9,766 IJ/termite (*H. sonorensis* Kemondji). These numbers are considerably lower than those found for other hosts such as the corn earworm (311,000 IJ/insect) or *G. mellonella* (200,000 IJ/insect) (Dutky *et al.*, 1964; Cabanillas & Raulston, 1994). Difference may be due to host size and/or host suitability to the EPN isolates, justifying the common use of *G. mellonella* as control host in several bioassays.

In southern Benin, citrus farmers grow crops for food (maize, cassava, bean, cotton, peanuts and soybean) in association with citrus plantation. This cultural practice supports the proliferation of insect pests and diseases. As result, *Trinervitermes occidentalis*, a grass-

finding termite often occurs with *M. bellicosus* in the same citrus orchard, and appears to provide substantial damage to e.g. maize. The nests of both termite species are sometimes found very close to each other in the field. To predict the spill-over effect of EPN on *T. occidentalis* in citrus orchards, I compared the susceptibility of both termite species to EPN isolates. The study demonstrated that caste size strongly influences the susceptibility of both termite species to EPN. Larger caste size allows the EPN to penetrate *M. bellicosus* more easily through natural openings (mouth, anus, spiracles) or, in case of heterorhabditids, by disrupting the soft cuticle of the host by its dorsal tooth (Bedding & Molyneux, 1982). According to Pervez *et al.* (2012), the rate of penetration of EPN into the host insect can be used as a measure of host susceptibility. Soldiers of *M. bellicosus* (13.2-18.6% IJ penetration) were the most susceptible followed by workers of the same species (5.2-8.8% IJ penetration) compared with soldiers (1.0 - 1.4% IJ penetration) and workers (1.4- 2.0% IJ penetration) of *T. occidentalis*. Selvan *et al.* (1993) suggested that one of the criteria for determining host suitability is the level of IJ reproduction following infection. The heavier and larger soldiers and workers of *M. bellicosus* enabled better multiplication of IJ per insect, as compared with the small soldiers and workers of *T. occidentalis*. As a consequence of these observations, the most suitable host for all tested EPN isolates/species was *M. bellicosus*.

The EPN repellent-dispersing assay did not show evidence that both *M. bellicosus* and *T. occidentalis* would be able to detect the presence of IJ of any tested EPN isolates/species at 962.5 IJ/cm^2. On the contrary, earlier studies on other termite species proved an EPN repellence effect. Yu (2009) revealed that the termite species *Heterotermes aureus* was repelled by *S. riobrave, S. feltiae, S. carpocapsae* and *H. bacteriophora* treated areas for up to 10 days at 250 IJ per gram of soil. However, the repellence threshold was found to vary among nematodes species. In the study undertaken by Wang *et al.* (2002), *H. indica* repelled *Reticulitermes flavipes* for up to 17 days at 362 nematodes /cm^3. Mauldin and Beal (1989) found that *S. carpocapsae* also repelled *R. flavipes*. To the best of my knowledge, my study seemed to be the first showing the non-repellence effect of EPN to termites. Future investigations using much higher IJ concentration are needed to confirm these findings. It was observed in my study that nematode dispersal occurred by infected termites (= phoresis). These findings, supported by the foraging strategy (cruisers) of tested EPN isolates, are of paramount importance in the success of the bio-control of termites in the field. Termites that can detect the presence of EPN, may respond by removing nematode from their body or

walling off nematode-killed termites (Fujii, 1975; Epsky & Capinera, 1988; Wang *et al.*, 2002).

An integrated pest management tactic involving the use of native EPN in conjunction with pesticides may play an important role in managing *M. bellicosus* populations in citrus. This is feasible when the pesticide does not negatively affect the utilized EPN. Therefore, I assessed the influence of the commonly used pesticides (fipronil, sulphur and glyphosate) in citrus in southern Benin on EPN isolates. In an earlier study, the nematode *S. carpocapsae* (Wesier) has been shown to offer much potential for tank-mixing with chemical insecticides for turf pest management (Alumai & Grewal, 2004). Research involving *S. feltiae* has also shown considerable success in using the nematode in conjunction with chemical insecticides registered in the UK for the control of both *Bemisia tabaci* on both tomato and verbena foliage (Cuthbertson *et al*., 2003) and *Liriomyza huidobrensis* (Blanchard) on glasshouse lettuce crops (Head *et al*., 2000). The results of my study demonstrated that EPN isolates can tolerate the tested pesticides, but only at a given exposure time, regardless of the pesticide concentration ranging from 0.5- to 2-fold field-recommended concentration. However, the increase of exposure time and pesticide concentration had a negative impact on the infectivity of EPN against *G. mellonella* larvae. Generally, a 2.3-10.5 h exposure of EPN to tested pesticides may lead to 50% mortality of exposed IJ populations. It was shown previously in Chapter 4 that the EPN needed 3-6 h to invade 50 % of exposed termite populations. Care should then be paid if one decides to apply EPN in conjunction with these pesticides. Sequential instead of simultaneous application of EPN and agrichemicals might be the better choice in an IPM system (Head *et al*., 2000). Anyway, future implementation of synchronous application of EPN and tested pesticides must be supported by field experiments, while results of laboratory experiments cannot be transferred uncritically into conditions which hold for the environment.

Studies carried out by Lüscher (1961) and Ruelle (1964) showed that termite nest temperatures are constant at around 30°C for *M. bellicosus*, with a mean annual fluctuation of less than 1°C. However, it may reach a peak of 40°C inside the nest of *T. occidentalis*. Both these insects also require moisture, not only from the surrounding soil, but also from their food and air (relative humidity around 70 to 80%) (Watson, 1969). As tested EPN will be applied to termite nest environments, information on the influence of soil temperature and moisture on their infectivity is of great interest. Laboratory bioassays revealed that increasing temperature (27 to 37°C) has a negative impact on the virulence of EPN isolates to workers of

T. occidentalis. *Heterorhabditis indica* Ayogbe1 is known to infect its host at temperatures above 29°C (Grewal *et al.*, 1994); however, from my study, *H. sonorensis* Azohoue2 recorded better results by inducing the highest termite mortality at 27, 30, 33 and 35°C compared to *H. indica* Ayogbe1 for the same temperature range. Unlike soil temperature, the increase of soil moisture up to 20 % (w/w) did not negatively influence the virulence of EPN isolates to workers of *T. occidentalis*; however, 5% soil moisture content badly affected the virulence of tested EPN isolates to *T. occidentalis*. Low soil moisture may induce a quiescent dehydration-survival state of EPN isolates (Lewis, 2002). This suggests that during application and post-application, desiccation of soil should be avoided or soil moisture should at least be maintained at a level sufficient for minimal nematode activity.

Due to the side effects of chemical pesticides and the repetitive failures recorded with the farmers' traditional practices to manage termites, Beninese farmers are eager to find effective and safer methods to control termites. In order to facilitate the adoption of EPN by farmers, four pilot farmers were chosen and directly involved in the process of the implementation of the current programme designed to use EPN to control termites. Each pilot farmer is a member of a cooperative of at least 20 members and will train and disseminate their knowledge and knowhow to train the other members of their cooperative. They were invited in the laboratory to be informed about EPN and trained on the production and formulation of *G. mellonella* larvae (Figure 7.1). There were interactive exchanges in the midst of the training, mainly on the EPN application strategies with greatest successes inside termite nests.

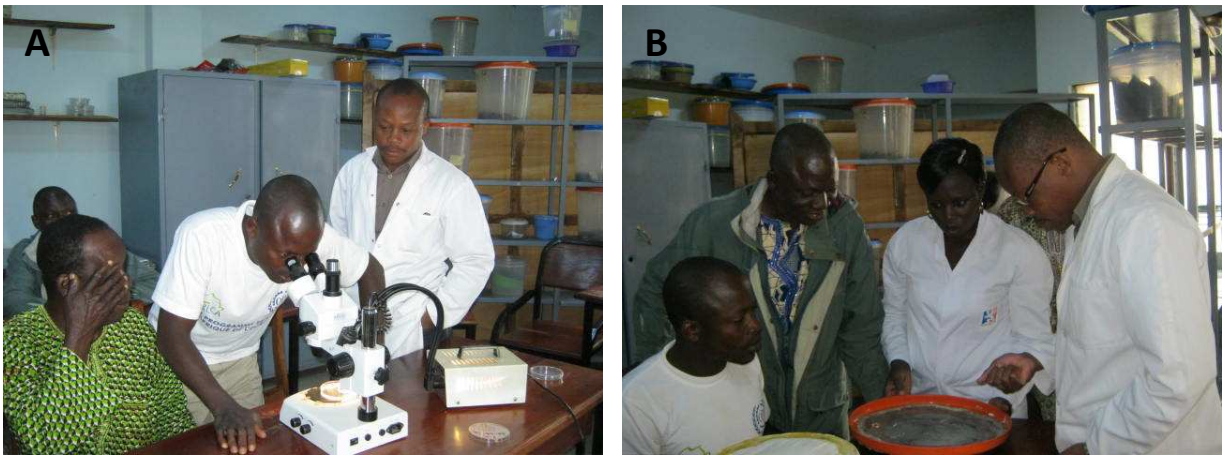

Figure 7.1 Training of farmers on entomopathogenic nematodes (A) and production of larvae of *Galleria mellonella* (B).

As an important amount of *Galleria* larvae were needed, beyond their training on *Galleria* larvae production, pilot farmers were also provided with materials to start their own production of *Galleria* larvae (Figure 7.2). The produced larvae will be collected periodically from farmers; and infected with a chosen EPN isolate for further applications on their own fields. A monitoring schedule will be defined together with farmers to control the efficacy of EPN.

Figure 7.2 Farmers received material to produce larvae of *Galleria mellonella*

Agricultural technologies are developed and promoted. There is a need to integrate indigenous knowledge about pest management techniques into the scaling-up process in order to improve farmers' pest management practices (Nyeko *et al.*, 2004). The majority of the Beninese citrus growers cannot afford synthetic chemicals and keep using their traditional practices, which consist of the application of wood ash or engine oil following the destruction of the aboveground nest. In most cases, *M. bellicosus* reconstructs its nest after some weeks/months. In my view, integrated pest management is essentially the result of a knowledge-based decision-making process. To assess the potential of EPN isolates to control the underground *M. bellicosus* populations, a field evaluation was carried out in orchards of the pilot farmers. The pilot farmers were involved in the implementation and the monitoring of the field trials (Figure 7.3). The main objective of this study was to "embed EPN in farmers' control practice to develop an environmentally-sound and sustainable termite control alternative to synthetic chemicals at cottage industry level". In this respect, based on the interactive exchanges with

pilot farmers during the training, EPN-infected cadavers replaced the ash of wood or engine oil used by farmers, in field evaluation of tested EPN.

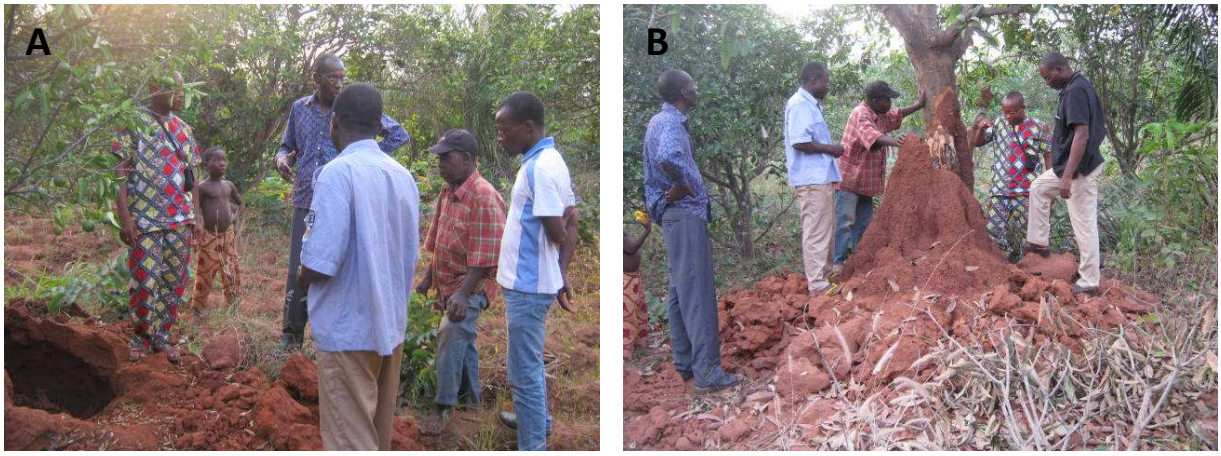

Figure 7.3 Farmers watching the effect of entomopathogenic nematodes on the underground population of *Macrotermes bellicosus*. A = Treated nest and B = untreated control nest.

As a result, the entire underground populations of 71% or 60 % of the termite nests treated with respectively *G. mellonella* larvae infected with *H. sonorensis* Azohoue2 or *H. indica* Ayogbe1 were controlled at 70 days after application. In spite of nematode persistence observed in all treated nests, both tested EPN isolates failed to control the underground termite populations of some treated nests. These failures were hard to explain. Current field EPN assessment is the first out of a series foreseen for the development of a sustainable termite control using EPN. A reliable explanation may be found in further investigations which take into account additional parameters such as: the fluctuation of soil moisture and temperature inside individual nest, the occurrence of biotic antagonists (e.g. nematophagous fungi) during the experiments, and the repellent effect of EPN as well as the grooming behaviour of tested termites in field conditions.

Current thesis is the first comprehensive study on the use of EPN to control termite pests, mainly *M. bellicosus*, in Benin. The research presented has led to useful information on the distribution of EPN in southern Benin. It has also led to a better understanding of the biological trait of Beninese indigenous EPN and their interaction with *M. bellicosus*. Furthermore, the research also justifies future successes in the use of EPN in the development of a suitable integrated termite management as an alternative to synthetic chemicals at cottage

industry level in Benin. However, there are still questions to be answered in order to improve the understanding of the use of EPN in biocontrol of *M. bellicosus* in Benin.

Further investigations will have to address the following aspects:

1- Development of a suitable formulated-EPN infected cadaver in order to develop an effective formulation providing extended shelf life, stability of product from transport to application and ease of handling;

2- Monitoring of soil temperature and moisture fluctuation and investigation of possible occurrence of biotic antagonist inside individual nest in order to put light on the EPN failures observed in the field evaluation;

3- Check repellence effect of EPN on termite in field conditions in order to confirm laboratory bioassay.

4- Find a susceptible host other than *G. mellonella* larvae in order to assure EPN maintenance in dry season. *G. mellonella* hardly reproduces in dry season;

5- Investigating long-term storage of EPN to standardize the density and temperature in order to avoid sudden death of the IJ of EPN during storage.

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SUMMARY

The biocontrol potential of native entomopathogenic nematodes (EPN) towards termites in citrus in southern Benin was explored. Surveys for EPN were conducted for the first time in Benin (Chapter 2). The prospected sites were also surveyed for the occurrence of termite species. The most frequent termite species found in citrus orchards belong to the subfamily Macrotermitinae, *viz.*, *Amitermes guineensis*, *Ancistrotermes crucifer*, *Trinervitermes occidentalis* and *Macrotermes bellicosus* (ESCiP-Benin, 2011); the latter being the most dominant and damaging.

A total of 84 sites were prospected from 6 communes, *viz*. Atlantique, Couffo, Mono, Oueme, Plateau and Zou. *Galleria mellonella* larvae-baiting method was used to extract EPN. Out of 280 soil samples collected, 32 were positive for the occurrence of EPN. Morphological and molecular identification showed that all the EPN isolates belong to the genus *Heterorhabditis*; 29 isolates were identified as *Heterorhabditis sonorensis* and the remaining (3) isolates as *H. indica*. This is the second record of *H. sonorensis* since its description from the Sonora dessert in Mexico; and so, the first in Africa. The surveys indicated that the occurrence of EPN isolates was associated with habitat, vegetation and soil types. However, no significant difference was observed in ecological trends of both EPN species taken individually.

The recovered isolates (29 isolates of *H. sonorensis* and one of *H. indica*) were subjected to a series of bioassays in order to select superior isolates for biological control. They were first screened for their pathogenicity towards the target pest, *Macrotermes bellicosus* (Chapter 3). The majority of the isolates (73 %) killed more than 80 % of the exposed termites after three days at a concentration of 50 infective juveniles (IJ)/termite. Tolerance of the IJ of EPN isolates to heat, desiccation and hypoxia was assessed as well as the effect of these environmental extremes on the IJ pathogenicity. The IJ of EPN isolates/species differed significantly for their ability to tolerate tested environment stresses. However, none of them was found to be superior to the others for all of the tested factors. Six isolates of *H. sonorensis* (Zagnanado, Kpedekpo, Akohoun, Djidja2, Kassehlo and Zoundomey) proved to be the most adapted to heat (40°C, 8 h) with 53% < IJ survival < 73 %. Nine of the *H. sonorensis* isolates (Kpanroun, Setto1, Zoundomey, Azohoue1, Azohoue2, Kemondji, Akohoun, Kassehlo and Dan) proved to be the most adapted to desiccation (25% glycerol, 8 h) 53% < IJ survival < 73 %. Most of them (70%) proved to tolerate hypoxic conditions with 52% < IJ survival > 82%.

The pathogenicity of IJ against *Galleria mellonella* larvae was significantly affected by heat (30-63.3 %), desiccation (60-78.3 %) and hypoxia (21.7-48.3 %).

Beside tolerance to environmental extremes, the EPN isolates were also screened for their ability to migrate towards *M. bellicosus* in sand column assays (Chapter 4). Both *H. sonorensis* and *H. indica* isolates were found to exhibit a cruise foraging strategy. However, only three isolates of *H. sonorensis* isolates (Yokon, Aglali and Djidja1) were able to cause 100% mortality to *M. bellicosus* at 20 cm from inoculated point after 72 h. These isolates were followed by five other isolates of *H. sonorensis* (Hessa2, Ze3, Azohoue2, Zoundomey and Kemondji) and the only *H. indica* isolate (Ayogbe1) inducing mortality between 86 and 94 % to *M. bellicosus* in the same conditions. These latter nine isolates were then subjected to virulence parameters including time-parasitism response (IT_{50}), dose-mortality response (LC_{50}), and time-mortality response (LT_{50}). Their potential to produce offspring inside *M. bellicosus* was also evaluated (Chapter 4). No significant differences were observed among selected EPN isolated in term of LC_{50} values. However, the isolates were found to differ in IT_{50} and LT_{50} values. Based on these two latter parameters, the isolate Ze3 (IT_{50} = 3.35 h; LT_{50} = 23.30 h) appeared to be the most virulent followed by the isolate Azohoue2 (IT_{50} = 3.67 h; LT_{50} = 29.43 h). In respect of reproduction potential, the *H. sonorensis* isolate Yokon was the most reproductive in *M. bellicosus* with 20213 IJ/termites followed by *H. sonorensis* Ze3 with 19368 IJ/termite.

In chapter 5, host suitability of EPN isolates was assessed by comparing the susceptibility of the target pest *M. bellicosus* and *T. occidentalis*, a termite species often found in the same field as *M. bellicosus*. The repellent effect of EPN on both termite species was also examined. Two isolates of *H. sonorensis* (Ze3 and Azohoue2), one of *H. indica* (Ayogbe1) and an undescribed isolate of *Steinernema* sp. (Bembereke) were tested. The latter isolate was obtained from an extra survey and was added to broaden EPN species diversity. Based on data on virulence, penetration rate, and number of emerging IJ from termite, worker and soldier castes of *M. bellicosus* appeared more susceptible to all tested EPN species/isolates than worker and soldier castes of *T. occidentalis*. The most suitable host for all tested EPN isolates/species was *M. bellicosus*, having the largest soldier and worker castes.

The possibility of applying EPN isolates in conjunction with the commonly used pesticides including insecticide (fipronil), fungicide/insecticide (sulphur) and herbicide (glyphosate) in citrus in southern Benin was explored under laboratory conditions (Chapter 6). The Time

(ST_{50}) at which 50% of a population of IJ of EPN survived after exposure to these pesticides at field-recommended concentration was estimated. This study showed differential sensibility of EPN isolates to tested pesticides. The ST_{50} values varied significantly from 3.3 h (*Steinernema* sp. Bembereke) to 10.5 h (*H. sonorensis* Azohoue2) for glyphosate; from 3 h (*Steinernema* sp. Bembereke) to 10.4 h (*H. indica* Ayogbe1) for fipronil; and from 2.3 h (*H. indica* Ayogbe1) to 7.4 h (*Steinernema* sp. Bembereke) for sulphur. Moreover, increase exposure time was found to be detrimental to IJ pathogenicity.

The influence of soil temperature and moisture on IJ is of paramount importance for nematode persistence the following hours after application. A study was carried out to assess virulence of the four aforementioned EPN isolated as affected by soil temperature and moisture regimes (Chapter 6). It showed that efficacy of all tested EPN isolates/species was negatively influenced by soil temperature ranging from 27 to 37°C, the greatest impact being observed at 37°C. Unlike soil temperature, the increase of soil moisture up to 20 % (w/w) did not reduce the virulence of EPN isolates to workers of *T. occidentalis*; however, a moisture content of 5% did reduce the virulence.

In the field experiment (Chapter 6), 2-week EPN-infected *G. mellonella* larvae were used to control the underground population of *M. bellicosus*. The results showed that the entire population of 70% or 60% of treated nests were controlled by *H. sonorensis* azohoue2 or *H. indica* Ayogbe1, respectively.

In conclusion, current PhD research showed for the first time the occurrence of EPN in Benin. The bioassays and field experiments clearly demonstrated the potential of indigenous EPN for the control of the termite pest *M. bellicosus*. Further investigations are still needed for the development at cottage industry level and practical application of suitable formulated EPN-infected insect cadavers as a new cost-effective, environmentally-sound and sustainable termite control alternative to synthetic chemicals.

SAMENVATTING

De mogelijkheid om entomopathogene nematoden (EPN) te gebruiken als biologische antagonisten van termieten in citrus in Zuid-Benin, werd bestudeerd. Voor het eerst werden het voorkomen van EPN in Benin in kaart gebracht (Hoofdstuk 2). Tegelijkertijd werden ook de aanwezige termietensoorten verzameld en geïdentificeerd. In citrusboomgaarden behoren de meest frequent voorkomende soorten tot de subfamilie Macrotermitinae; het betreft *Amitermes guineensis, Ancistrotermes crucifer, Trinervitermes occidentalis* and *Macrotermes bellicosus* (ESCiP-Benin, 2011). Deze laatste is de meest overheersende en ook de meest schadelijke soort.

In totaal werden 84 plaatsen in 6 provincies (communes), nl. Atlantique, Couffo, Mono, Oueme, Plateau en Zou bemonsterd. De extractie van EPN gebeurde met *Galleria mellonella* larven. In 32 van de 280 bodemstalen werden EPN gevonden. De identificatie gebeurde aan de hand van morfologisch en moleculair onderzoek en toonde aan dat alle geëxtraheerde soorten tot het genus *Heterorhabditis* behoorden; 29 isolaten werden geïdentificeerd als *H. sonorensis* en de overige 3 als *H. indica*. *H. sonorensis* werd voor het eerst sinds zijn originele beschrijving uit de Sonora woestijn in Mexico, teruggevonden; het is dus een eerste vermelding voor Afrika. Het onderzoek toonde aan dat het voorkomen van EPN nauw verbonden was met het type habitat, vegetatie en bodemtype. Indien de EPN-soorten afzonderlijk werden beschouwd, bleek dat wat de ecologische factoren betreft, geen significant verschil te bestaan tussen beide EPN soorten.

Voor de selectie van het meest geschikte EPN-isolaat als biologische antagonist, werden de 32 EPN isolaten van *H. sonorensis* en *H. indica* onderworpen aan een reeks bio-toetsen. Vooreerst werden ze gescreend voor hun afdodingsefficiëntie voor de termiet *M. bellicosus* (Hoofdstuk 3). De meerderheid van de isolaten (73%) doodde na drie dagen meer dan 80% van de termieten die blootgesteld werden aan 50 infectieve juvenielen (IJ) per termiet. De tolerantie van IJ voor (1) hoge temperaturen, (2) uitdroging en (3) zuurstofgebrek (hypoxia) werd onderzocht, evenals het effect van deze extreme omgevingsfactoren op hun virulentie. De IJ van EPN isolaten/soorten verschilden significant wat betreft hun vermogen om de onderzochte omgevingsstressfactoren te tolereren. Toch was geen enkele van deze geteste extreme omgevingsfactoren superieur t.o.v. de andere. Zes isolaten van *H. sonorensis* (Zagnanado, Kpedekpo, Akohoun, Djidja2, Kassehlo en Zoundomey) bleken het best aangepast aan hoge temperatuur (40°C, 8 h) met 53% < IJ overleving < 73 %. Negen isolaten

van *H. sonorensis* (Kpanroun, Setto1, Zoundomey, Azohoue1, Azohoue2, Kemondji, Akohoun, Kassehlo en Dan) waren het best aangepast aan uitdroging of dessicatie (25% glycerol, 8 h) 53% < IJ overleving < 73 %. De meeste isolaten (70%) bleken tolerant aan toestanden met verminderd zuurstofgehalte met 52% < IJ overleving> 82%. De virulentie van IJ voor *G. mellonella* larven was significant beïnvloed door hoge temperatuur (30-63.3%), uitdroging (60-78.3%) en hypoxia (21.7-48.3%).

Naast het onderzoek naar de tolerantie van EPN isolaten voor extreme omgevingsfactoren, werd ook het migratievermogen onderzocht van de isolaten naar de termiet *M. bellicosus* in een zandkolomtest (Hoofdstuk 4). Bij beide EPN soorten, *H. sonorensis* en *H. indica* werd een 'cruise foeragering' strategie waargenomen. Na 72 uur, konden slechts drie *H. sonorensis* isolaten (Yokon, Aglali en Djidja1) een 100%-mortaliteit van *M. bellicosus* veroorzaken op 20 cm van het inoculatiepunt. Vijf andere isolaten van *H. sonorensis* (Hessa2, Ze3, Azohoue2, Zoundomey and Kemondji) en slechts één *H. indica* isolaat (Ayogbe1) veroorzaakten onder dezelfde omstandigheden tussen 86 en 94 % mortaliteit van *M. bellicosus*. Van deze negen isolaten werden dan onderworpen aan virulentie-parameters met inbegrip van tijd-parasitisme response (IT_{50}), dose-mortaliteitsresponse (LC_{50}), en tijd-mortaliteitresponse (LT_{50}). Het vermogen om zich te kunnen vermenigvuldigen in de termiet *M. bellicosus* werd ook onderzocht (Hoofdstuk 4). Er werd geen significant verschil in LC_{50}-waarden vastgesteld tussen de geselecteerde EPN isolaten maar hun IT_{50}- and LT_{50}-waarden waren wel verschillend. Gebaseerd op deze laatste twee parameters bleken de isolaat Ze3 (IT_{50} = 3.35 h; LT_{50} = 23.30 h) de meest virulente, gevolgd door isolaat Azohoue2 (IT_{50} = 3.67 h; LT_{50} = 29.43 h). Voor wat betreft het voorplantingsvermogen, was *H. sonorensis* isolaat Yokon het meest reproductieve isolaat in *M. bellicosus* met 20213 IJ/termiet, gevolgd door *H. sonorensis* Ze3 met 19368 IJ/termiet.

De gastheergeschiktheid voor EPN-isolaten werd nagegaan door de vatbaarheid van doelgerichte schadelijke insecten *M. bellicosus* en *T. occidentalis* te testen. *T. occidentalis* kwam vaak samen voor met *M. bellicosus*. Het afstotingseffect van EPN voor beide termietensoorten werd ook bestudeerd. Twee isolaten van *H. sonorensis* (Ze3 en Azohoue2), één van *H. indica* (Ayogbe1) en een niet-beschreven isolaat van *Steinernema* sp. (Bembereke) werden getest. Het *Steinernema* isolaat werd gevonden tijdens een latere survey naar EPN en werd aan deze studie toegevoegd om de diversiteit van de isolaten te verhogen. Op basis van gegevens over virulentie, de graad van penetratie en het aantal IJ dat de termiet verlaat, bleken de kasten werkers en soldaten van *M. bellicosus* gevoeliger te zijn voor de andere geteste EPN

soorten/isolaten dan deze van *T. occidentalis*. De meest geschikte gastheer van alle geteste EPN isolaten/soorten was *M. bellicosus*; deze hebben de grootste soldaten en werkers kasten heeft.

Hoofdstuk 6 bespreekt het laboratoriumonderzoek dat de mogelijkheden onderzocht van het toepassen van EPN-isolaten samen met de meest gebruikte pesticiden, zoals fipronil (insecticide), zwavel (fungicide/insecticide) en glyfosaat (herbicide), in citrus in Zuid-Benin. Het tijdstip werd bepaald waarop 50% van een populatie van IJ overleeft (ST_{50}) na blootstelling aan deze pesticiden bij concentraties die worden aangeraden voor veldtoepassing. Deze studie toont het verschil in gevoeligheid voor de geteste pesticiden tussen de isolaten. De ST_{50} waarden variëren significant van 3.3 h (*Steinernema* sp. Bembereke) tot 10.5 h (*H. sonorensis* Azohoue2) voor glyphosaat; van 3 h (*Steinernema* sp. Bembereke) tot 10.4 h (*H. indica* Ayogbe1) voor fipronil en van 2.3 h (*H. indica* Ayogbe1) tot 7.4 h (*Steinernema* sp. Bembereke) voor zwavel. Het verhogen van de tijd waarin de EPN isolaten werden blootgesteld aan deze pesticiden was ongunstig voor hun virulentie.

De invloed van de bodemtemperatuur en -vochtigheid is van het allergrootste belang voor het overleven van de IJ in de uren volgend op de toepassing. Hun invloed op de afdodingsefficiëntie (virulentie) van de vier vermelde EPN isolaten werd onderzocht (Hoofdstuk 6). De werkzaamheid van alle geteste EPN isolaten werd negatief beïnvloed door een bodemtemperatuur tussen 27 en 37°C, waarbij het grootste effect werd waargenomen bij 37°C. In tegenstelling tot de bodemtemperatuur, verminderde de stijging van de bodemvochtigheid tot 20% (w/w) niet de virulentie van EPN isolaten voor werkers van *T. occidentalis*; een daling van de vochtigheid met 5% reduceerde echter hun virulentie.

In het veldexperiment (hoofdstuk 6) werden 2 weken EPN-geïnfecteerde *G. mellonella* larven gebruikt om de ondergrondse populatie van *M. bellicosus* te bestrijden. De resultaten toonden aan dat de hele populatie van 70% of 60% van de behandelde nesten bestreden werd met *H. sonorensis* azohoue2 of H indica Ayogbe1, respectievelijk.

Het onderzoek toonde aan dat entomopathogene nematodensoorten voorkomen in Benin. De bio-toetsen en veldexperimenten onderstrepen de mogelijkheden van het gebruik van inheemse EPN voor de bestrijding van de schadelijke termieten zoals *M. bellicosus*. Verder onderzoek is nodig voor het ontwikkelen van de productie van EPN als antagonist van termieten. Eveneens moet het onderzoek leiden tot de praktische toepassing van EPN onder de vorm van een geschikte, geformuleerde EPN-geïnfecteerde insectkadaver als een nieuw,

rendabel, milieuvriendelijk en duurzaam bestrijdingsmiddel als alternatief voor synthetische chemicaliën.

Curriculum Vitae

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